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STUDIES OF CERTAIN NON-PELLICLE-FORMING VINEGAR BACTERIA[1]

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The group of vinegar bacteria was first studied extensively by Hansen (1894), Beyerinck (1898), and Henneberg (1898) who independently classified them into a number of species basing their classification largely on morphological and physiological variations. These species were later reclassified by Janke (1916) into groups and sub-groups based principally on morphological differences and on one physiological characteristic, namely, that of the ability to grow in Hoyer's mineral solution. More recently Bergey *et al.* (1923), applying the International Rules of Botanical Nomenclature to bacteriology, attempted to bring about a more systematic arrangement and reclassified the entire group of vinegar bacteria under the genus *Acetobacter* with eleven subordinate species. The bacteria in these different classifications are described as possessing the common characteristic of forming at some period of their development a pellicle or film on the surface of suitable liquid media. Although species have been classified as to the thickness or the quality of the pellicle, no attempt seems to have been made to differentiate non-pellicle-from pellicle-forming vinegar bacteria or to describe the former as separate species. In 1924 Kluyver and De Leeuw (1924) described a new species of vinegar bacteria, *Ace. suboxydans*, characterized as non-pellicle-forming and as being able to produce crystals of calcium-oxygluconate on yeast extract agar

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supplemented with 2 per cent calcium carbonate and 2 per cent glucose. The following year, during the course of a study of vinegar making from cull apples the writer (1925) reported the isolation of vinegar bacteria which never form a pellicle, yet produce large quantities of acetic acid from alcoholic fermented cider. This discovery and that of Kluyver and De Leeuw suggested further investigation with regard to the proper place of these bacteria in the systematic classification, under the genus *Acetobacter* or otherwise, and also with regard to their comparative economic value.

The work here reported deals with the morphology, cultural characteristics, and physiology of these organisms and to a limited extent with their comparative ability to produce acetic acid in alcoholic fermented cider. A vigorous strain of *Ace. aceticum* was used in the last part of this study, primarily for the sake of comparison. The methods for pure culture study of bacteria as prescribed by the Society of American Bacteriologists were followed as closely as the nature of the organisms permitted. Yeast extract agar to which was added 2 per cent glucose, 2 per cent CaCO₃, and 10 per cent apple cider, or plain agar supplemented with 10 per cent apple cider were used because much better growth was secured on these media than on plain agar. The yeast extract was prepared from compressed yeast according to the method of Fred (1919). Eight strains of the non-pellicle-forming bacteria were used in this work, but only two of the most representative strains are included in this paper. The cultures used were as follows:

Ace. suboxydans—a culture supplied by Dr. Kluyver[2] of Delft, Holland.

Strains (a) and (b)—two strains of the non-pellicle-forming vinegar bacteria isolated from cider in 1925. These two strains differ slightly in morphology.

Ace. aceticum—a vigorous strain used for vinegar experiments in this laboratory.

I. MORPHOLOGY

Form: *Ace. suboxydans* and *Strain (b)*. Rods, short to medium with rounded ends. They are arranged singly and in chains or filaments in liquid cultures.

Strain (a). Most rods are short, some being nearly spherical. They are arranged singly and in chains or filaments in liquid cultures.

Size: *Ace. suboxydans* and *Strain (b)*. The two species are quite similar in size. In forty-eight hour cultures and in old cultures from both liquid and agar media most of the individual cells measure from 0.8 to 1.0 in width by 1.0 to 2.0 in length.

Strain (a). Most cells measure from 0.6 to 1.0 in width by 0.8 to 1.5 in length.

Motility: None are motile in twenty-four hour or forty-eight hour-broth cultures.

Staining reactions: All are Gram-negative and fail to stain with iodine.

Spore formation: Both young and old cultures fail to show spores and do not resist heating at 85°C. for ten minutes.

II. CULTURAL CHARACTERISTICS

Agar streak: The growth of each of the three organisms is moderate, slowly spreading, slightly raised, and of a dull grayish luster. The topography is smooth and the growth opaque. Characteristic odors are absent. The consistency is butyrous and the medium unchanged in color. Old cultures of *Ace. suboxydans* produce calcium oxy-gluconate crystals on yeast extract cider agar to which calcium carbonate and glucose has been added. The other types never produce crystals.

Agar plate colonies: Ace. suboxydans grows rapidly, forms circular colonies on yeast extract cider agar and both circular and irregular colonies on plain cider agar. The surface is smooth, the elevation convex, the edges entire except on plain cider agar where a few colonies are lobate or erose. The internal structure is amorphous. Young colonies produce a clear ring on yeast extract cider agar, supplemented with calcium carbonate and

glucose. Old cultures produce crystals of calcium oxy-gluconate on this same medium.

Strains (*a*) and (*b*) grow rapidly, forming circular colonies on yeast extract cider agar and broth, circular and irregular colonies on plain cider agar. The surface is smooth, the elevation convex, the edges entire except on plain cider agar where a few colonies are lobate. The internal structure is amorphous. Young colonies produce a clear ring on yeast extract cider agar supplemented with $CaCO_3$ and glucose. Old cultures never produce crystals of calcium oxy-gluconate.

Nutrient broth: The three organisms leave the surface of the broth clear without pellicle formation. A slight clouding caused by a small amount of rather compact sediment results when shaking the broth tubes. Characteristic odors are absent.

III. PHYSIOLOGY

Temperature relations: The three organisms grow best at 25° to 30°C.

Chromogenesis: No pigment is formed on agar or broth cultures.

Relation to oxygen: There is no growth in agar shakes, but some growth occurs in liquid cultures covered by a layer of paraffin oil. The organisms are aerobic.

Milk: No change appears in litmus milk and apparently no growth occurs.

Relation to nitrogen: Ace. suboxydans and *Strain* (*b*) do not use nitrogen in the form of ammonia as evidenced by the absence of growth in Hoyer's solution—Visser 'T. Hooft (1925).

Strain (*a*) grows in Hoyer's solution and is able to use nitrogen in the form of ammonia.

Fermentation: Ace. suboxydans is catalase positive and produces acids in beef broth and yeast extract from the following sugars and alcohols: Glucose, mannitol, sucrose, lactose, ethyl alcohol, propyl alcohol, butyl alcohol, glycol.

Strains (*a*) and (*b*) are catalase positive and produce acid in beef broth and yeast extract from the following sugars and alcohols: Glucose, sucrose, ethyl alcohol, propyl alcohol.

To give a more definite idea of the acid formation of these

organisms the quantitative production of each is recorded in table 1. The acid was determined by titrating 1 cc. of the medium with N/10 NaOH using phenolphthalein as indicator. The oxidation of the various sugars and alcohols was carried on in large test tubes containing 15 cc. of media. The sugars were added in 5 per cent quantities and the alcohols in 3 per cent portions. Both the yeast extract and beef broth media were adjusted to the neutral point of phenolphthalein before inocula-

TABLE 1

Fermentation of sugars and alcohols in beef broth and yeast extract

Cubic centimeters of N/10 NaOH required to neutralize 1 cc. of media after four and six weeks growth

Ingredients used	BEEF BROTH								YEAST EXTRACT							
	Ace sub-oxydans		Strain b		Strain a		Ace. aceticum		Ace. sub-oxydans		Strain b		Strain a		Ace. aceticum	
	4 weeks	6 weeks	4 weeks	6 weeks	4 weeks	6 weeks	4 weeks	6 weeks	4 weeks	6 weeks	4 weeks	6 weeks	4 weeks	6 weeks	4 weeks	6 weeks
Glucose	2.5	3.4	2.8	2.9	3.0	3.3	2.6	3.2	2.3	3.2	0.6	0.8	1.6	2.0	0.9	1.3
Mannitol	0.7	1.0	0.1	0.2	0.1	0.3	0.0	0.1	0.6	1.4	0.2	0.2	0.1	0.3	0.0	0.0
Sucrose	1.7	2.1	1.4	2.1	1.4	2.4	0.2	0.3	0.7	2.0	0.8	0.7	0.8	1.0	0.2	0.4
Lactose	0.5	1.0	1.0	2.0	0.0	1.0	0.0	0.0	0.6	1.2	0.0	0.2	0.0	1.0	0.0	0.0
Ethyl alcohol	2.1	2.8	3.0	3.4	1.3	1.8	1.4	1.9	0.8	1.7	1.0	1.5	0.7	0.8	1.0	1.5
Propyl alcohol	0.3	0.6	1.2	1.6	2.5	3.4	2.2	3.0	1.0	1.9	0.3	0.5	1.3	1.3	1.5	2.1
Glycol	1.0	1.5	1.1	4.1	0.1	2.0	0.8	1.2	0.8	1.0	1.5	0.6	0.6	0.6	0.4	0.9
Butyl alcohol	0.4	0.8	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.9	0.0	0.0	0.0	0.0	0.0	0.0

tion with the organisms. When only very small amounts of acid were produced they were not considered because they might have resulted from impurities present in the sugars and alcohols which were used as obtained from the supply houses without previous recrystallization or redistillation. The data presented are averages of duplicate test tubes.

In any classification of the various species of the acetobacter group attention must be given to the physiological as well as to the morphological and cultural characteristics. The physiological characteristics in particular deserve special consideration because there exist wide and specific differences of oxidation

powers among the various species. Advantage has been taken of these differences in the classification of Bergey *et al*, who divided the genus acetobacter into five groups according to the oxidation of specific sugars and alcohols. They further arranged the eleven species among these groups according to differences in morphology and differences in the chemical composition and tenacity of the pellicles. If these principles were to be applied to the organisms studied here *Ace. suboxydans* would probably come under the fourth group with regard to oxidation of sugars and alcohols. But its morphological differences and especially its rare quality of producing crystals of calcium oxy-gluconate in certain media would segregate it from that group and make it necessary to classify it as a separate species. On the other hand *Strains* (*a*) and (*b*) have oxidation powers similar to the third group of Bergey *et al.*, but neither produces the cartilagenous pellicle or gives the cellulose reaction of the species in that group. Furthermore they differ widely from each other with regard to the utilization of nitrogen, hence each would have to be classified as a separate species. But it is questionable whether such a procedure would be justified. Judging from recent contributions to the knowledge of vinegar bacteria it seems that the physiological and morphological differences are subordinate to the more fundamental biochemical variations of the bacterial protoplasm. These factors have not received much attention in the classification of Bergey. There is also some difference of opinion regarding the selection of some characteristics of the species in this classification. Visser 'T. Hooft (1925) in a critical biochemical study of the acetobacter groups says:

I cannot entirely agree with the American Committee in regard to the description of the genus because of the fact that it is certainly unjust to characterize all of the vinegar bacteria as non-motile. The most varying species may appear in very motile forms. Therefore, I should like to change the identification at this point as either motile by means of polar flagella or non-motile. At the same time it would also be advisable to change the word *certain* other carbonaceous compounds to *many* other carbonaceous compounds.

Although I am well aware that a key for the determination of species

does not have to take into account the fundamental relationships of these species, it must be observed that the characteristics used in this key have been selected in an unfortunate manner. First, it is entirely unjust to state that all the bacteria described form acetic acid from the substrata in question. This can easily be explained by the fact that Bergey *et al.* were satisfied with rearranging a table compiled by Henneberg in 1898 without taking into consideration that when Henneberg observed acid production it was not necessarily always acetic acid. It is definitely shown from this misconception how little the exact meaning of the chemical and decomposition processes were understood. It is now evident from the investigations discussed in this paper that these characteristics are of an incidental character and are entirely unfit for a true identification.

The criticisms offered for the species accepted by Henneberg can also be applied to the remainder of the ten species of Bergey *et al.* Furthermore the absence of synonyms of all the species described by Beyerinck and Hoyer is very conspicuous.

In the light of the information now available on the vinegar bacteria, thanks to the investigations of Visser 'T. Hooft, many of his criticisms seem justified, though it should first of all be recognized in justice to Bergey *et al.* that their reclassification of these bacteria was merely a serious attempt to bring order out of chaos based on the information available in the literature and was not a systematic classification based on their own critical investigations of the organisms. A truly orderly and permanent classification must eventually be the outcome of exhaustive studies of groups of organisms by independent or coöperating investigators. Visser 'T. Hooft's contribution to the acetobacter group is unquestionably of much value in obtaining this end. Therefore, a careful consideration of his proposed classification is in order.

He recognizes the fact that any systematic classification of bacteria must take account of the more essential morphological as well as physiological characteristics. Under the more essential morphological characteristics he considers, first, the cell structure, second a well established life cycle, as for example, endospore formation, and third, the position of the flagella if they are present. Concerning the physiological characteristics he points

out as essential, first, the way in which the cell provides for its energy needs, that is the chemistry of the decomposition process, and second the affinity of the bacterial protoplasm for hydrogen. He concedes that practically all the investigators who study the vinegar bacteria do so primarily for the purpose of making use of the ability of these organisms to oxidize to a partial extent the energy foods at their disposal. A typical example is the manufacture of vinegar, where it is of the utmost significance that the manufacturer be thoroughly familiar not only with the rapidity and capacity of vinegar production, but also with the further oxidation of this product by his bacteria. Therefore, it is important to classify the vinegar bacteria from the standpoint of their oxidation power and it is with this end in view that Visser 'T. Hooft has proposed his new classification. He distinguishes the following biochemical types and species.

A. Catalase negative vinegar bacteria.
.   I. Bacteria which oxidize gaseous hydrogen.
                              1. *Ace. peroxydans* n. sp.
        II. Bacteria which do not oxidize gaseous hydrogen.
B. Catalase positive vinegar bacteria
        I. Bacteria which grow in Hoyer's solution.
                              2. *Ace. aceticum* Beyerinck
       II. Bacteria which do not grow in Hoyer's solution.
            1. Bacteria which form acids and carbonates on alcohol calcium carbonate agar plates.
                              3. *Ace. rancens* Beyerinck
            2. Bacteria which form only acids on alcohol calcium carbonate agar plates.
                a. Bacteria which form gluconic acid from glucose calcium carbonate agar plates and later oxidize to calcium carbonate.
                              4. *Ace. xylinum* Brown
                              5. *Ace melanogenum* Beyerinck
                b. Bacteria which form gluconic acid from glucose calcium carbonate agar plates and later oxidize to calcium oxy-gluconate.
                              6. *Ace. suboxydans* Kluyver and DeLeeuw

The author of this proposed classification believes that these distinctions are of sufficient importance to justify the subdivision of the vinegar bacteria under these six headings. However, he does not mean that upon further morphological and physiological

proof it may not be desirable or even necessary to subdivide into several species those bacteria now included under each heading. But he points out that under this arrangement any future systematic classification of the vinegar bacteria must take into account the existence of the six species listed above which are already sufficiently distinguished.

This classification undoubtedly gives a clear biochemical distinction which is largely based on the oxidation powers of the species. The variations of these oxidation powers are believed to be the direct result of specific differences in the hydrogen affinity of the bacterial protoplasm. No thought seems to have been given to the possibility of modifying these oxidation powers through adaptation of the bacterial cell to unfavorable conditions or media. That such modifications are possible in certain cases was demonstrated in this study by adapting *Strain* (*a*), first, to a modified Hoyer's medium and finally to the standard Hoyer's solution. This was done in the following manner.

Hoyer's solution was modified by using acetic acid instead of phosphoric acid to clear it. Then 1.5 per cent agar was added to a portion of the solution. Plates prepared with this agar medium were streaked and tubes with the modified solution were inoculated with *Strains* (*a*) and (*b*). No growth occurred in the solution but a scanty development of *Strain* (*a*) appeared on the plate. This growth was later transferred to the modified solution in which it developed poorly. Then it was transferred back to the solid medium and better growth was realized. The process was repeated several times until finally this strain succeeded in growing in modified Hoyer's solution. The experiment was successfully repeated with standard Hoyer's solid and liquid media. After growth was well established and acid was formed in Hoyer's solution, the culture was used to inoculate alcoholic fermented cider in which it formed acetic acid readily and showed all the characteristics of the original *Strain* (*a*).

This illustrates the fact that rigorous tests may sometimes be necessary to establish definitely certain characteristics in bacteria. How far bacterial variations can be carried on is problematic. If similar biochemical reactions result from similar protoplasmic

compositions of bacterial cells as Kluyver and Donker (1924) contend, it is evident that reactions caused by bacteria are restricted to the same limits as other biochemical reactions. This conception would reduce bacterial variations to relatively narrow limits. But the character of the reactions may also be misunderstood at times. At first thought, the fact that certain species of vinegar bacteria are able to use ammonia nitrogen and others are not may not always appear to be a property of oxidation. Yet, Visser 'T. Hooft (1925) ascribes the ability of *Ace. aceticum* to

TABLE 2

Comparative oxidation powers of various species of vinegar bacteria

NAME OF BACTERIA	GLUCOSE	MANNITOL	SUCROSE	LACTOSE	ETHYL ALCOHOL	PROPYL ALCOHOL	BUTYL ALCOHOL	GLYCOL	CATALASE	HOYER'S SOLUTION	YEAST EXTRACT AGAR +2 PER CENT CaCO$_3$ +2 PER CENT C$_2$H$_5$OH	YEAST EXTRACT AGAR +2 PER CENT CaCO$_3$ +2 PER CENT GLUCOSE	PELLICLE
Ace. aceticum.	+	−	+	−	+	+	−	−	+	+	A.C.	A.C.	+
Strain (a). ...	+	−	+	−	+	+	−	−	+	+	A.C.	A.C.	−
Ace. rancens	+		(+)		+	+	(+)	(+)	+	−	A.C.	A.C.	
Strain (b).. ..	+	−	+	−	+	+	−	(+)	+	−	A.C.	(A.C.)	−
Ace. suboxydans	+	(+)	+	(+)	+	+	(+)	(+)	+	−	A.	A.Cr.	−

(+) = small amounts; A = acid; C = calcium carbonate; Cr. = calcium oxygluconate crystals.

use ammonia nitrogen in the presence of alcohol to its strong oxidation power. Various factors like these point to the need for detailed and accurate investigations to establish bacterial characters which are to be used for systematic classification. Nevertheless the proposed classification of Visser 'T. Hooft has many advantages. It brings out important biochemical characteristics heretofore ignored. It overlooks some of the less important morphological characteristics and by so doing combines several of the species of Bergey *et al.* into one. This is perhaps justifiable, because it tends to simplify the classification

and makes the division lines more distinct. However, it seems that the possibility of bacterial variation by adaptation has not received sufficient attention. It is possible that careful investigations along this line would result in a further rearrangement of some of the species. At least it would certainly establish their characteristics more definitely, a thing that is wanting in most of the classified species. Considering the proposed classification as a whole, however, it is an improvement over former classifications and seems to be more acceptable than that of Bergey *et al.* Under it *Strains* (a) and (b) would not be entered as separate species, but *Strain* (a) would perhaps be a variety of species *Ace. aceticum* and *Strain* (b) a variety of *Ace. rancens.* The characteristics which determine this arrangement are given in table 2 together with the characteristics of species *Ace. aceticum,* *Ace. rancens* and *Ace. suboxydans* as given by Visser 'T. Hooft.

Judging from the evidence secured in this study it seems logical not to consider *Strains* (a) and (b) as separate species as would result from following the classification of Bergey *et al.* but to place them as varieties of species *Ace. aceticum* and *Ace. rancens* respectively according to the proposed classification of Visser 'T. Hooft.

THE COMPARATIVE EFFICIENCY IN VINEGAR PRODUCTION OF THE BACTERIA STUDIED

Since the ultimate economic value of the acetobacter group lies in the production of acetic acid it is important to know how the various species differ in this respect. A preliminary experiment was carried out to determine this difference. Sixteen 500-cc. portions of fresh cider were sterilized in the autoclave in 1-liter Erlenmeyer flasks and cooled to a room temperature which varied from 21° to 25°C. This temperature was maintained throughout the experiment. After cooling, the flasks were inoculated with a pure culture of *S. ellipsoideus* and six weeks later, when the alcoholic fermentation was completed, quadruplicate flasks were again inoculated with equal amounts of a heavy suspension of each of the following organisms. *Ace. suboxydans,* *Strains* (a) and (b) and *Ace. aceticum.*

Two of each of the quadruplicate flasks were stoppered with cotton plugs and allowed to ferment in this condition. The remaining duplicate flasks were attached to absorption towers containing approximately $N/5$ KOH solution to collect the escaping gases. This system was connected to a suction pump by means of which a constant stream of sterile CO_2 free air was drawn over the surface of the cider. The purpose of this arrangement was to increase the air supply to the liquid and to ascertain the amount of CO_2 resulting from the metabolic processes of the vinegar bacteria. Before admitting the air to the flasks it was passed first, through a concentrated solution of H_2SO_4 provided with glass beads to break up the air bubbles and to insure sterilization; second, through a tower filled with soda lime to absorb the CO_2; third, through a concentrated solution of KOH; and finally, through a flask partially filled with sterile beef broth to saturate the air with moisture and to ascertain its sterility. This procedure proved to be satisfactory as no growth was observed in the beef broth.

Titrations of all ciders and KOH solutions were made once a month. The ciders were titrated with $N/10$ KOH using phenolphthalein as indicator, according to the official method for vinegar, and the results were calculated in per cent of acetic acid. Ten-cubic centimeter samples taken under aseptic conditions were used for this purpose. The KOH solutions in the absorption towers were subjected to the double titration with $N/10$ HCl, using thymol blue and brom phenol blue as indicators. The results were arbitrarily calculated in milligrams of CO_2. The data recorded in tables 3 and 4 represent the average results of the acid and CO_2 determinations of duplicate flasks.

Under the conditions of this experiment *Ace. suboxydans* was not as good a vinegar producer from cider as either of *Strains* (*a*) and (*b*) or as *Ace. aceticum*. Both *Strains* (*a*) and (*b*) formed more acid and produced it much faster than either of the two other species named. The data also seem to indicate that increased aeration retarded acid production perceptibly during the first two months and that there was a tendency to the contrary towards the end of the experiment. However, this observa-

tion may be incidental, and no definite explanation can be given for it until further work on a more extended scale has been accomplished.   That it was incidental in the case of *Ace. aceticum* can readily be explained by the fact that in one of the duplicate flasks no pellicle was formed until the fourth week after inoculation.   This made the results of the first and second determinations extremely low.   But leaving aside this discrepancy and

TABLE 3

Per cent of acetic acid production in alcoholic fermented cider by various species of vinegar bacteria in aerated and cotton stoppered containers

ORGANISM	AERATED BOTTLES				COTTON STOPPERED BOTTLES			
	1 month	2 months	3 months	4 months	1 month	2 months	3 months	4 months
Ace. suboxydans	1 53	1 89	3 33	4 16	3 48	3 69	3 90	4.53
Strain (a)	2.24	5 21	6 14	6 73	4 98	5 19	5 31	5.96
Strain (b)	2.71	3 90	5 09	6 06	4 71	5 09	5.55	5.98
Ace. aceticum	0 24	1 46	5 10	6 04	1.44	4 08	4 23	4 77

TABLE 4

Gaseous substances resulting from the metabolic processes arbitrarily calculated in milligrams of CO_2

ORGANISM	FIRST MONTH	SECOND MONTH	THIRD MONTH	FOURTH MONTH	TOTAL IN 4 MONTHS
Ace. suboxydans	444	167	431	409	1451
Strain (a)	431	508	613	682	2234
Strain (b)	756	438	581	463	2238
Ace. aceticum	536	645	882	394	2457

viewing the results in general from the economic standpoint of the vinegar manufacturer it is evident that the two strains of the non-pellicle-forming bacteria would be selected in preference to the other two, because of the fact that they are capable of forming more acetic acid and of producing it much faster than either of the others.

According to the old belief that the activities of vinegar bacteria are confined to the oxidation of alcohols and sugars to acetic acid without the liberation of CO_2 it would appear that the data

given in table 4 are unsound. However, this belief is no longer acceptable, since Söhngen (1914) observed that in a culture medium of yeast extract to which 20 per cent glucose was added vinegar bacteria produced not only large quantities of gluconic acid and acetic acid, but also alcohol to the amount of 1 per cent. Thus the possibility of CO_2 production as a result of the metabolic processes of these bacteria was demonstrated, provided the proper substratum was used. In this connection Visser 'T. Hooft (1925) stated that as the vinegar bacteria become farther removed from the immediate surface of the substrata they have less access to free oxygen and are compelled to provide for their oxygen requirements in some other way. This he claims to be possible by a series of intramolecular and intermolecular dehydrogenation and hydrogenation processes by means of which glucose is broken down to acetaldehyde and CO_2 and finally to alcohol and acetic acid. Under the conditions of this experiment it is hardly conceivable that purely chemical action causing hydrolysis of the starch or cellulose materials present in the sediments of the cider could result in the formation of a sufficient amount of glucose to account for all the CO_2 liberated. It might be argued, on the other hand, that the yeast cells present in the substrata were the cause of CO_2 production. This would only be possible during the first stages of acetic acid fermentation, for according to Le Fevre (1924) 0.5 per cent acetic acid interferes seriously with the growth of yeast and 1.0 per cent is almost prohibitive. Consequently it would follow from this contention that after the first month of acetic acid fermentation the yeast cells ceased to be a factor in this experiment at least. This viewpoint is all the more convincing because of a similar production of CO_2 observed in a previous experiment (1926) which was carried on under somewhat different and less controlled conditions for a period of one year. Such a constant liberation of small amounts of CO_2 over such long periods of time during the acetic acid fermentation of ciders suggests that vinegar bacteria are probably capable of utilizing considerable quantities of carbonaceous matter of a more complex structure than sugars and alcohol. This was apparent from the fact that the CO_2 given off must

have resulted in a large measure from other sources than the oxidation of acetic acid to its end products of CO_2 and water, because this liberation of CO_2 was invariably associated with a corresponding increase in acetic acid. It seems that facts like these serve as a strong indication that certain important metabolic processes of the vinegar bacteria are as yet unknown and, therefore, are deserving of special attention and careful study. If they were thoroughly understood and properly correlated with the known characteristics of the vinegar bacteria they might prove to be of marked significance in industrial vinegar production.

SUMMARY

1. Comparative morphological and physiological studies of several strains of a non-pellicle-forming vinegar bacterium isolated from cider and of *Ace. suboxydans* Kluyver and DeLeeuw (1924) suggest two new species in addition to *Ace. suboxydans* according to the classification of Bergey *et al.*

2. A critical study was made of the proposed classification of Visser 'T. Hooft who considers the oxidation power of the genus Acetobacter a function of the bacterial protoplasm and uses it as a basis for systematic classification. Under this classification, which seems to be more acceptable than that of Bergey *et al.*, the non-pellicle-forming vinegar bacteria isolated from cider would not be entered as new species, but *Strain* (*a*) would be a variety of species *Ace. aceticum* and *Strain* (*b*) a variety of species *Ace. rancens*.

3. A preliminary experiment on the efficiency of various species of vinegar bacteria including *Strains* (*a*) and (*b*), *Ace. suboxydans* and *Ace. aceticum* proved that the two non-pellicle-forming strains formed more acetic acid in cider and produced it much faster than either of the other two species.

4. The liberation of small amounts of CO_2 associated with a corresponding increase in acetic acid during the acetic acid fermentation suggested that vinegar bacteria may be able to utilize considerable quantities of carbonaceous matter of a more complex structure than sugars and alcohols.

REFERENCES

BEYERINCK, M. W. 1898 Cent. f. Bakt., II abt., 4, 209–16.

FRED, E. B., PETERSON, W. H., AND DAVENPORT, AUDREY 1919 Jour. Biol. Chem., 39, 347–383.

HANSEN, E. C. 1894 Compt. Rend. Carlsberg Lab., Copenhagen, 3, 182.

HENNEBERG, W. 1898 Cent. f. Bakt., II abt., 4, 14.

JANKE, A. 1916 Cent. f. Bakt., II abt., 45, 1–50.

KLUYVER, A. J., AND DELEEUW, F. J. G. 1924 Tydschr. v. Vergelyk. Geneesk, Leiden, 10, 170–181.

KLUYVER, A. J., AND DONKER, H. J. L. 1924 Versl. Kon. Akad. A'dam., 33, 896.

LE FEVRE, E. 1924 Farmers' Bul. 1424, U. S. D. A.

SÖHNGEN, N. L. 1914 Folia Microbiologica, 3, 151.

VANDECAVEYE, S. C. 1925 Wash. Agr. Exp. Sta. Bul. 196, 15.

VANDECAVEYE, S. C. 1926 Wash. Agr. Exp. Sta. Bul. 202, 3–9.

VISSER 'T. HOOFT 1925 Diss. Boekhandel & Drukkery, W. D. Meinema, Delft, Holland, 84–123.

THE RELATION OF THE HYDROGEN ION CONCENTRATION OF EGG WHITE TO ITS GERMICIDAL ACTION[1]

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INTRODUCTION

The so-called bactericidal or germicidal property of the white of hens' eggs has attracted but little attention in the past ten years. Before that time, various investigators from 1890 onward have recorded studying this subject, some reporting a positive, others a negative action. Wurtz (1890) seems to have been the first to suggest that egg white possessed a germicidal power; others who confirmed his results and carried their investigations further were Scholl (1893) Horowitz (1902) Turró (1902) Laschtschenko (1909) Rettger and Sperry (1912) and Sperry (1913). On the other hand, Parascandole (1893) and others state that egg white does not possess such a property, but that bacterial growth can take place in it. Some investigators found a difference in the germicidal action of the egg white depending upon the organism used, the rate of seeding, treatment of the albumin, etc. Hadley and Caldwell (1916) in their extensive review of the literature conclude "in summary, perhaps all that can be said at the present time is that certain species of bacteria, when placed in albumin mixtures, survive but a short time."

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Of the theories advanced to explain the bactericidal property of egg white, probably the first ones were those which were based on the assumption of a high osmotic pressure or lack of food materials. Laschtschenko (1909) believed that he disproved both of these theories when he showed that the white still possessed its germicidal action toward *Bacillus subtilis* when diluted with bouillon, water, or physiological salt solution. He advanced the theory that the bactericidal action was due to the presence of an enzyme of a proteolytic nature. As proof of this he found that egg white which had been heated to 55° to 60°C. for thirty minutes still possessed its germicidal action, while egg white which had been heated for the same length of time to 65° to 70°C. failed to show such an action. Other investigators have confirmed the results of Laschtschenko, and Rettger and Sperry (1912) have even suggested egg white heated to 65° to 70°C. as a good culture medium.

Bainbridge (1911), working with pure egg albumin solutions, found that when bacteria were grown in such solutions no multiplication took place when the seeding was large; small seedings did increase slightly, however, and he assumed from this that there was present an insufficient amount of non-protein nitrogenous material in egg albumin for marked bacterial growth, but that the small trace that was present permitted the slight growth observed when small seedings were used. By adding a few drops of broth or an impure albuminose solution to the pure egg albumin, he found that rapid multiplication resulted, even with the large seedings; no pure albumin, however, was broken down

Rettger and Sperry (1912) were inclined to explain Bainbridge's results on the ground that pure and unchanged proteins may play the part of antiseptics or germicides. They report in another paper, Sperry and Rettger (1915), that certain obligate aerobes, facultative anaerobes, and obligate anaerobes were unable to develop in a medium which contained pure egg albumin as the only source of carbon and nitrogen. When a small amount of peptone was added to the medium, the organisms developed and decomposed the egg albumin. They state, "The egg white even appeared to have an antiseptic, and, in a measure, bactericidal

action on aerobes.   This property was undoubtedly not due to the egg albumin, but to other agencies in the egg."

EXPERIMENTAL

It is unfortunate that most of the investigators who have worked on the germicidal property of egg white failed to record in their published work the exact age of the eggs when examined. Eggs are spoken of as "fresh," "reasonably fresh," "newly laid," etc., and in the majority of the contributions no adjective at all describes the egg white that was used for testing out the bactericidal property.   It has been found by Healy and Peter (1925) and, independently, through extensive experiments carried out in this laboratory by Dr. Powell and one of us (Sharp), that the whites of eggs decrease in hydrogen ion concentration, under some conditions, very rapidly after being laid, approaching as a limit a pH of approximately 9.5.   This phenomenon is due to diffusion of carbon dioxide from the egg white.

The results obtained in this laboratory show that the change in the hydrogen ion concentration of the white of eggs is very rapid if the eggs are kept at room temperature freely exposed to the air.   The change is less rapid at lower temperatures but still takes place.   The white of the egg may change as much as 1.0 pH units in twenty-four hours at room temperature and in six days the change may be as much as 1.8 pH units.   Egg white obtained from eggs within an hour or two after they were laid and thoroughly beaten to mix the thin and thick white was found in our experiments to have a pH of about 8.2.   The actual pH of the white of fresh hens' eggs is much lower than 8.2, probably about pH 7.6, but special precautions must be taken in determining the hydrogen ion concentration of the white in order to obtain the lower values.   Eggs after aging for one week in a well ventilated place at room temperature will usually have a white with a pH of about 9.4.   This is a considerable change in hydrogen ion concentration and should be great enough to affect the growth of bacteria.   In table 1 will be found the limiting maximum pH for the growth of a few organisms as reported in the literature.   McClendon and Medes (1925) give a list of 24

different bacteria and the range of hydrogen ion concentration through which they will grow. The values for the. limiting maximum pH for growth range from pH 7.6 to 10.0 with an average of 8.1.

Eggs which have been kept at room temperature for two days in air have whites with a pH a little above 9.0. A glance at table 1 will indicate that most of the bacteria would be killed if placed in the white of such a two-day-old egg, while they would grow in the white of an egg one hour old. It should be remembered, however, that the limiting hydroxyl ion concentration for the growth of bacteria varies with the other components of the medium.

TABLE 1

The limiting maximum pH for the growth of various organisms as reported in the literature

ORGANISM	MAXIMUM HYDROXYL ION CONCENTRATION	OBSERVER
	pH	
Bacillus subtilis . . .	9 4	Itano (1916)
Diplococcus pneumoniae	8 6	Avery and Cullen (1919)
Bacterium typhosum	8 6	Schoenholz and Myer (1921)
Bacterium aerogenes	8 4	Cohen and Clark (1919)
Bacterium dysenteriae	8 5	Cohen and Clark (1919)
Bacterium alkaligines	9 7	Cohen and Clark (1919)
Proteus vulgaris	8 8	Cohen and Clark (1919)
Lactobacillus bulgaricus	8 0	Cohen and Clark (1919)
Corynebacterium diphtheriae	8 2	Bunker (1917)

It may be safely stated that the eggs used by most previous workers had reached a pH of 8.8 to 9.0, as an egg a day or so old is considered "fresh" by the average person. In many cases they may have been several days' old; for example, Hadley and Caldwell (1916) in their investigation of the bacterial content of fresh eggs say that "During one year of the work, the eggs were never more than eight days old at the time of the examination." Such eight-day-old eggs would probably have a white with a pH of approximately 9.4, which is above the maximum pH range for most bacterial growth as indicated by the literature.

In the light of this knowledge of the change in the hydrogen ion concentration of egg white with age and in view of the really high pH value which may be reached, it is possible to explain much of the apparently conflicting evidence of previous investigators on the assumption that they were studying egg whites of different pH values. It is possible that those investigators who reported that egg white had no germicidal properties used as their experimental material relatively fresh egg white (pH 7.6 to 8.4), while those investigators who reported a germicidal action used more alkaline egg white (pH 9.2 to 9.5).

From Parascandole's (1893) description of his experiments in which he found that 9 different organisms grew in egg white, we are led to suspect that he used egg white of low pH value. He took freshly laid eggs, washed them with alcohol, immersed them in mercuric chloride solution for 10 minutes, and covered them with paraffine until he was ready to remove the egg white. Experiments in this laboratory have shown that if eggs, the white of which has a low pH, are coated with paraffine the increase in pH on aging in air is inhibited. From the account of this investigator's procedure it seems highly probable that he actually did work with egg white of a low pH value approximating that of the egg as laid, and this accounts for his obtaining growth of bacteria in egg white.

Maurer (1911) also probably investigated the germicidal action of egg white toward *Bacterium coli* before the pH of the egg white had increased appreciably for he obtained growth. We have found that this organism is able to grow in egg white with a pH corresponding to that of the fresh egg white, but is killed at pH values corresponding to that of egg white from eggs which have been aged in air for a few days.

Rettger and Sperry (1912) used eggs which were "reasonably fresh" in studying the germicidal action of the egg white, and it is therefore, highly probable that the egg white had attained a high pH value. That this was the case is also indicated in our experimental work, for we investigated two of the organisms— *Bacillus cereus* and *Bacillus megatherium*, which Rettger and Sperry found were sensitive to the germicidal action of egg white.

We found these organisms sensitive to the germicidal action of alkaline egg white corresponding to the pH of aged eggs while these same organisms exhibited growth in egg white having a pH approaching that of eggs one hour old. Turró (1902) made the (to him) surprising observation that the white of the fresh egg had a weaker germicidal power than the white of aged eggs. This observation is exactly in agreement with the postulate which led us to start this investigation.

When we call our experimental material fresh egg white, we mean egg white obtained from eggs which had been laid less than two hours. The actual pH of this material varies somewhat, depending on its age and treatment.

The literature also shows conclusively that different bacteria may vary in their sensitiveness to the germicidal action of egg white.

Sherman and Curran (1924) have shown the desirability of using young cultures of organisms in the period of rapid growth when determining gemicidal properties, in order to remove any influence of the "lag period." Therefore, in this work three-hour-old cultures were used unless otherwise stated.

In order to determine the effect of the hydrogen ion concentration on the rate of growth of bacteria in egg white, a series of test tubes was arranged containing equal volumes of fresh, well mixed egg white, ranging in pH from 5.0 to 10.0 The pH was adjusted by adding varying amounts of normal hydrochloric acid and sodium hydroxide. The tubes were all made up to the same volume with sterile distilled water. All hydrogen ion concentration determinations reported in this paper were made at 25°C. using the hydrogen electrode. In no case did the dilution due to acid or alkali exceed ten per cent of the volume of the egg white used. One cubic centimeter of a culture of *Pseudomonas pyocyaneus* (an organism which seems adapted to egg white, having been isolated from an infected egg white in this laboratory, and having been reported as present in eggs by several workers) was added to the tubes, mixed well, and incubated for six hours at 37°C. The check on the original seeding and the count after the six-hour period were made by the plate method.

In all cases the original count was determined by adding the same volume of the inoculating culture to a water blank and plating at once.   This procedure was necessary because some organisms were killed so rapidly by the egg white that a substantial decrease in numbers took place before the organisms could be distributed in the egg white and a plate poured.   The results are shown in tabular form in table 2 and graphically in figure 1.

From this table we see that egg white of pH 9.4 showed a marked decrease, while egg white of pH 8.7 showed a substantial

TABLE 2

The growth of Pseudomonas pyocyaneus in egg white of various hydroxyl ion concentrations.   Incubation, after inoculation, at 37°C. for six hours, with an initial count of 1,410,000 bacteria per cubic centimeter

HYDROGEN ION CONCENTRATION		COUNT AFTER 6 HOURS (BACTERIA PER CUBIC CENTIMETER)
At start	After 6 hours	
pH	*pH*	
4 94	4 97	32,000,000
5 53	5 68	49,000,000
6 09	6 46	28,000,000
6 42	7 02	22,000,000
6 66	7 30	24,000,000
7 05	7 61	21,000,000
7 39	7 91	14,000,000
7 99	8 32	11,000,000
8 74	8 76	12,000,000
9 37	9 33	409,000
9 45	9 37	119,000
9 77	9 76	0
10 11	9 99	0
10 31	10 19	0
10 62	10 52	0

increase in numbers of bacteria over the original seeding.   At a pH of 9.7 there is a decided germicidal effect, all the organisms being killed.   This experiment was repeated with cultures of *Bacillus subtilis, Pseudomonas fluorescens, Serratia marcescens, Proteus vulgaris, and Bacterium coli* in the same manner and incubation continued for six hours, the initial count per cc. and the count after incubation being determined by the plate method. The results are given in table 3.
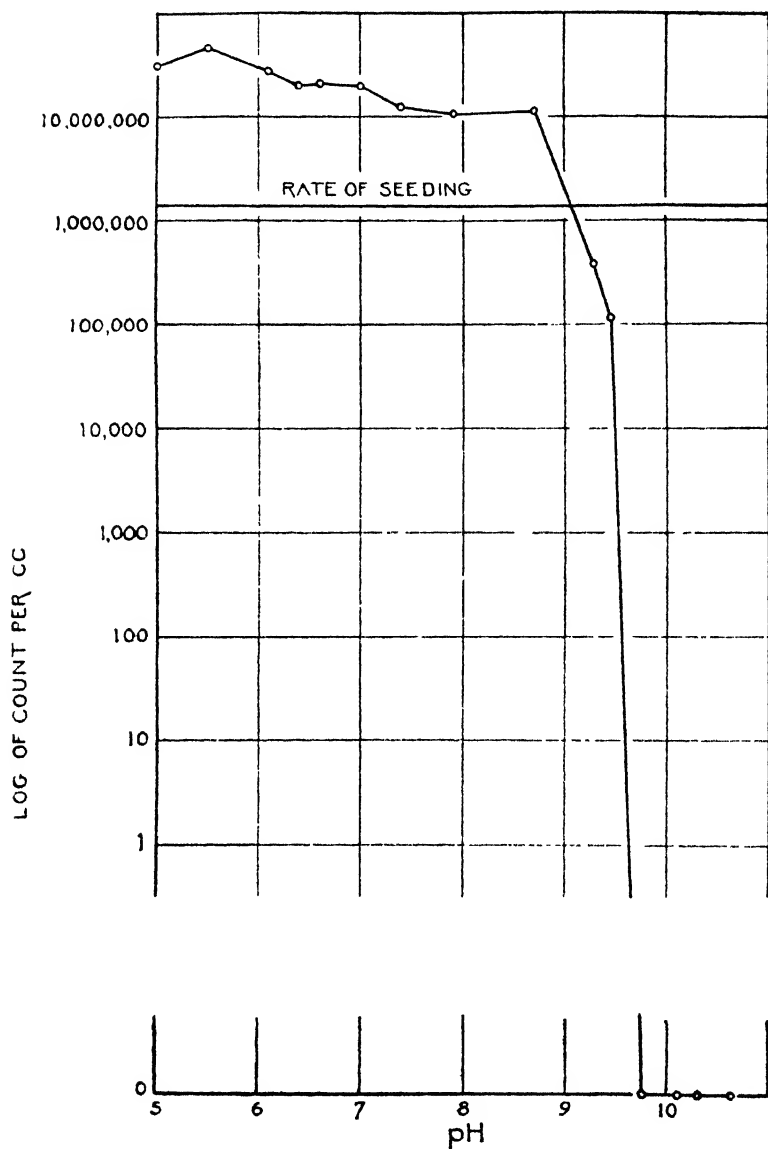
FIG. 1. THE RELATION OF THE HYDROGEN ION CONCENTRATION OF EGG WHITE
TO ITS GERMICIDAL ACTION TO PSEUDOMONAS PYOCYANEUS

Plate counts were made after incubating the inoculated egg white at 37°C.
for six hours.

As *Bacillus subtilis* in table 3 was the only spore producer, and as it apparently was killed very rapidly, other spore producing

TABLE 3

Egg white adjusted to various hydrogen ion concentrations and inoculated with Bacillus subtilis, Pseudomonas fluorescens, Serratia marcescens, Proteus vulgaris and Bacterium coli, incubated for six hours at 37°C. and the number of bacteria per cubic centimeter determined by the plate method before and after incubation

HYDROGEN ION CONCENTRATION	BACILLUS SUBTILIS PER CUBIC CENTIMETER	PSEUDOMONAS FLUORESCENS PER CUBIC CENTIMETER	SERRATIA MARCESCENS PER CUBIC CENTIMETER	PROTEUS VULGARIS PER CUBIC CENTIMETER	BACTERIUM COLI PER CUBIC CENTIMETER
pH					
5 34	<1,000	C	C	C	C
6 75	<1,000	C	C	C	C
8 28	<100	C	C	C	C
9 47	<100	13,000	<100	<100	19,000
10 65	<10	<10	<10	<10	<10
Original count	1,210,000	23,200,000	10,200,000	8,100,000	8,400,000

C = countless; < = less than.

TABLE 4

Egg white adjusted to various hydrogen ion concentrations and inoculated with the following spore-producing organisms, incubated for six hours at 37°C. and the number of bacteria per cubic centimeter determined by the plate method before and after incubation

HYDROGEN ION CONCENTRATION	BACILLUS MEGATHERIUM PER CUBIC CENTIMETER	BACILLUS CEREUS PER CUBIC CENTIMETER	BACILLUS MYCOIDES PER CUBIC CENTIMETER
pH			
6 76		20,200,000	230,000
7 73	10,000	5,900,000	330,000
8 52	11,700	69,000	<100
9 57	670	25,000	<10
9 88	<10	<10	<10
Original count	5,000*	1,300,000	43,000

< = less than.
* Approximated.

organisms were subjected to the same treatment.   The data are given in table 4.

Tables 2, 3 and 4 all indicate the validity of our original as-

sumption, that the pH of the natural egg white may be an important factor in determining whether or not it will permit bacterial growth. It will be noted that the egg white with a pH of approximately 9.5 was germicidal to all of the organisms which we investigated. Tables 3 and 4 indicate that egg white at the higher pH values is slightly more germicidal to the spore forming than to the non-spore forming organisms. *Bacillus subtilis* was the only organism studied which was killed at all pH values.

Pseudomonas pyocyaneus is a suitable organism for use in determining the effect of pH on the germicidal properties of egg white, having the following advantages: (1) it forms round and distinct colonies which are easy to count, (2) it does not produce "spreaders," (3) it has an average hydroxyl ion concentration death point well within the pH range which may be attained by natural egg white, and (4) it has been shown to be present in egg white by many investigators, evidently growing where the pH is suitable. It was chosen, therefore, to study the relationship of the age of the eggs and the alkalinity of the egg white to the rate of bacterial growth. Eggs were placed in storage and at various intervals some were withdrawn and their germicidal property tested by the same method as that used in table 2, except that no adjustment of the pH was made.

The eggs were brought to the laboratory and divided into three lots. The first lot was stored in air; the second lot was given the "oil-dipped" treatment, which is practiced commercially at the present time. This "oil-dipped" treatment consisted in dipping the eggs for a specified length of time in a hot oil, especially prepared for the purpose of preserving eggs by a commercial concern. The third lot of eggs was stored in water glass, made up in the ratio of one part of water glass to twelve parts of water. The eggs were laid the same day and were in storage within two hours' time. The storage temperature was 30°C. Four eggs from each lot were withdrawn from time to time, the whites removed as aseptically as possible, and separately inoculated with a culture of *Pseudomonas pyocyaneus*, incubated at 37°C. for six hours and then plated. In table 5 will be found the results of this experiment. The pH values given are for the egg white of

TABLE 5

The changes in hydrogen ion concentration and germicidal power of the white of storage eggs, stored for various lengths of time at 30°C.

The eggs were divided into three lots, each lot being subjected to a different storage treatment, as (1) storage in air, (2) previously oil-dipped and then stored in air, (3) storage in water glass solution. On being removed from storage the eggs were opened and the whites separately inoculated at a known rate with *Pseudomonas pyocyaneus*, incubated for 6 hours and the count per cc. again determined. The hydrogen ion concentration was also determined on the whites of the eggs as they came from storage.

TREATMENT	EGG NUMBER	ORIGINAL EGGS — RATE OF SEEDING 3,390,000		FIRST DAY — RATE OF SEEDING 7,200,000		SECOND DAY — RATE OF SEEDING 2,100,000		FOURTH DAY — RATE OF SEEDING 1,500,000		EIGHTH DAY — RATE OF SEEDING 140,000		EIGHTEENTH DAY — RATE OF SEEDING 200,000		THIRTY-FIRST DAY — RATE OF SEEDING 3,090,000	
		Hydrogen ion concentration pH	Plate count after 6 hours per cubic centimeter	Hydrogen ion concentration pH	Plate count after 6 hours per cubic centimeter	Hydrogen ion concentration pH	Plate count after 6 hours per cubic centimeter	Hydrogen ion concentration pH	Plate count after 6 hours per cubic centimeter	Hydrogen ion concentration pH	Plate count after 6 hours per cubic centimeter	Hydrogen ion concentration pH	Plate count after 6 hours per cubic centimeter	Hydrogen ion concentration pH	Plate count after 6 hours per cubic centimeter
Stored in air	1	8 45	3,470,000	8 72	2,600,000	9 01	2,230,000	9 25	660,000	9 45	1,500	9 42	880	9 37	1,680,000
	2	8 42	3,100,000	8 64	3,360,000	9 13	24,200,000	9 42	88,000	9 45	6,600	9 48	340	9 37	568,000
	3	8 53	5,620,000	8 83	4,180,000	9 23	1,350,000	9 33	97,000	9 48	0	9 42	860	9 40	680,000
	4	8 57	5,900,000	8 79	5,180,000	9 11	3,900,000	9 37	93,000	9 52	400	9 26		9 26	28,100,000
Stored as oil-dipped	1			8 57	14,500,000	8 40	6,600,000	8 34	24,200,000	8 34	23,000,000	8 59	18,500,000	8.57	77,200,000
	2			8 52	11,500,000	8 52	6,200,000	8 32	5,200,000	8 45	31,500,000	8 69	32,700,000	8 72	54,800,000
	3			8 42	11,900,000	8 50	5,100,000	8 34	1,790,000	8 54	1,900,000	8 63	18,600,000		
	4			8 50	7,700,000	8 59	3,940,000	8 34	2,200,000	8 42	20,100,000	8.72	38,500,000	8.58	50,100,000
Stored in water glass	1			8 49	6,500,000	8 44	27,900,000	8 34	16,400,000	8 36	7,100,000	8 67	7,400,000	8 60	82,400,000
	2			8.54	33,500,000	8 49	4,700,000	8.42	2,500,000	8.36	1,070,000	8.67	10,200,000	8.67	62,400,000
	3			8 47	3,280,000	8 47	7,300,000	8 39	2,590,000	8 45	1,840,000	8 57	31,100,000	8 66	71,400,000
	4			8 49	2,280,000	8 50	7,400,000	8 40	2,080,000	8 39	3,200,000	8 56	9,000,000	8 63	62,000,000

each egg as it came from storage, not after the six-hour incubation period.

A glance at table 5 shows that the whites from the eggs stored in air rapidly became more alkaline, so that at the end of one week the pH had risen to 9.4. Closely related to this is the steadily increasing germicidal effect on the organism, the first day even showing slight evidence of inhibited growth, and a decided germicidal effect later. From table 5 it can be seen that after the high alkalinity has developed a slight decrease in the pH is noted in the eggs stored for the longer lengths of time. Tice (1911) followed the fall of the alkalinity of egg white during storage, by titration with twentieth normal sulphuric acid, and found that after seven months in storage there was a gradual decrease, approaching "a common locus" with the yolk, the acidity of which steadily decreased during storage at 32°F. At this point it is interesting to note that Sperry (1913) says in writing about the germicidal properties of eggs, "The whites of eggs which are 11 months old or more showed a tendency to lose these properties." This is completely in accord with the theory that the variation in the so-called germicidal action is really due to the hydroxyl ion concentration of the natural egg white.

Table 5 also shows that eggs stored in water glass and those which were oil-dipped did not show such a rapid rise in pH. The bacteria were shown to grow in the eggs from both of these treatments indicating the absence of the bactericidal property.

It was next deemed advisable to determine if "aged" egg white of naturally high pH, to which acid had been added to bring the pH down to that of fresh egg white, would support growth the same as the fresh egg white. This was done in the following manner: whites from eight eggs were taken, numbers 1, 2, 3, and 4 having a pH of 8.4 to 8.5, and numbers 5, 6, 7, and 8 from eggs which had been stored for five days at 30°C. The hydroxyl ion concentration of the whites from the last three eggs was decreased by adding varying amounts of normal hydrochloric acid until the pH approximated that found in the first four eggs. Egg white number 5 had no acid added to it. Each egg white was inoculated with a culture of *Pseudomonas pyocyaneus*, the rate of

seeding being 3,380,000 per cubic centimeter then incubated for six hours at 37°C. and the count per cubic centimeter again determined.

The results of this experiment show again the influence of the variation in the hydroxyl ion concentration on the so-called germicidal action. Egg whites nos. 1 to 4 with pH values between 8.42 and 8.57 showed 3 to 6 million bacteria per cubic centimeter after incubation as did egg whites nos. 6 to 8 (with pH value readjusted to 8.16 to 8.27. Egg white number 5, to which no

TABLE 6

Growth of various organisms in egg yolk to which alkali had been added to give a pH such as found in aged egg white

After inoculation the yolks were incubated at 37°C. for six hours The bacteria count per cubic centimeter was determined by the plate method before and after incubation

HYDROXYL ION CONCENTRATION	PSEUDOMONAS PYOCYANEUS PER CUBIC CENTIMETER	BACILLUS SUBTILIS PER CUBIC CENTIMETER	BACTERIUM COLI PER CUBIC CENTIMETER	SERRATIA MARCESCENS PER CUBIC CENTIMETER	BACILLUS MEGATHERIUM PER CUBIC CENTIMETER
pH					
5 88*	C	<1,000	C	C	5,100,000
8 97	15,400,000	72,000	18,000	C	<1,000
9 48	2,160,000	<1,000	<1,000	75,000	<100
9 90	<100	1,200†	<100	<100	<100
10 32	<10	1,400†	<10	<10	<10
Original count	1,280,000	491,000	2,820,000	1,440,000	157,000

* Original pH

† It was believed this was due to contamination.

C = countless; < = less than

acid had been added (pH 9.34), showed a marked decrease in the number of bacteria (460,000).

Experiments were carried out to see whether or not egg yolks and mixtures of whites and yolks could be made to exhibit bactericidal properties by the simple adjustment of the hydrogen ion concentration to pH 9.5 or 9.6; i.e., the pH found in the whites of aged eggs. In the first experiment various amounts of alkali were added to the yolks of fresh eggs in order to give such a pH. Into these adjusted mixtures were placed 1-cc. portions of cul-

tures of the following organisms: *Pseudomonas pyocyaneus, Bacillus subtilis, Bacterium coli, Serratia marcescens,* and *Bacillus megartherium,* the count before and after six hours of incubation at 37°C. being made by the plate method. The results are given in table 6.

This experiment was repeated using the yolk and white mixed together in the same proportions as found in eggs. The results are shown in table 7.

TABLE 7

Growth of various organisms in a mixture of egg white and yolk, in the proportion found in eggs, to which alkali had been added to give a pH such as found in aged egg white

After inoculation the mixtures were incubated at 37°C. for six hours. The bacteria count per cubic centimeter was determined by the plate method before and after incubation.

HYDROXYL ION CONCENTRATION	PSEUDOMONAS PYOCYANEUS PER CUBIC CENTIMETER	BACILLUS SUBTILIS PER CUBIC CENTIMETER	BACTERIUM COLI PER CUBIC CENTIMETER	SERRATIA MARCESCENS PER CUBIC CENTIMETER	BACILLUS MEGATHERIUM PER CUBIC CENTIMETER
pH					
7 15*	C	<1,000	C	C	30,000
9 11	1,260,000	<1,000	2,000	C	6,000
9 29	290,000	<1,000	5,500	6,100	<1,000
9.56	14,000	<100	600	<100	<100
10 59	130	<10	<10	<10	<10
Original count	10,120,000	799,000	9,080,000	8,080,000	131,000

* Original pH.
C = countless; < = less than.

The data in table 6 and 7 confirm the experiment of table 2, which indicates that the germicidal action of the egg white from normal aged eggs is due in part to the high hydroxyl ion concentration. The yolk, which previous investigators have indicated possessed no germicidal property, was shown to possess such a property when brought to the same hydroxyl ion concentration as is found in the aged egg white. This indicates that the germicidal property of normal egg white which has frequently been found by others can be largely explained on the basis that the limiting factor is the concentration of the hydroxyl ions.

Laschtschenko (1909) brought out some interesting facts in regard to the germicidal action of egg white.   He found that egg white diluted 1:1 with water, physiological salt solution, or bouillon, did not lose its germicidal action to *Bacillus subtilis*.   He found that dilutions of 1 part of egg white to 10 parts of water or physiological salt solution were germicidal to this organism,

TABLE 8

The effect on the hydrogen ion concentration of various dilutions of fresh and aged egg white, with water, physiological salt solution and bouillon

DILUTION		HYDROGEN ION CONCENTRATION	
Parts egg white	Parts water	Fresh egg white	Aged egg white
		pH	*pH*
1	0	7.82	9 46
1	1	7 98	9 48
1	5	7 91	9 69
1	10	7 82	9 72
Parts egg white	Parts salt solution		
1	0	7 82	9.46
1	1	8 04	9 33
1	5	8 05	9 37
1	10	7 96	9 34
Parts egg white	Parts bouillon		
1	0	7 82	9 46
1	1	7.87	8 17
1	5	7.48	7.28
1	10	7 36	7 00
0	1	6.84	6 84

while he obtained growth in one experiment and not in the other when he diluted 1:2 with bouillon.

The question of what effect dilution has upon the pH of egg white was next studied, and the results obtained are found in table 8.

Dilutions were made with fresh egg white having a pH of 7.82 and of egg white having a pH of 9.46, the latter obtained from eggs which had aged in air for some time.   Diluting egg white

of pH 7.82 with water apparently caused a slight decrease in the hydrogen ion concentration, for dilutions up to 1 part of egg white to 10 parts of distilled water; on the other hand diluting egg white with an original pH of 9.46 with water, gave solutions which were distinctly more alkaline than the original egg white before dilution.   Upon dilution of the egg white of pH 7.82 with physiological salt solution, there was a slight increase in the pH and then a decrease at the higher dilutions.   Diluting the more alkaline egg white with physiological salt solution apparently caused a very slight decrease in the pH.   Diluting with bouillon caused both samples of egg white to decrease in pH, the more

TABLE 9

Effect of heating egg white with a pH of 9.41 to 68°C. for thirty minutes on its germicidal properties

ORGANISM USED	COUNT PER CUBIC CENTIMETER		
	Initial	Unheated after six hours	Heated after six hours
Bacillus subtilis . . .	1,700,000	<100	Countless
Bacterium coli	7,200,000	328,000	Countless
Serratia marcescens	10,000,000*	71,000	Countless
Pseudomonas pyocyaneus .	2,670,000	808,000	Countless

* Estimated.
< = less than.

alkaline egg white decreasing the more, and at a dilution of 1 part of egg white to 10 parts of bouillon, this sample of alkaline egg white actually had become the more acid.   Laschtschenko (1909) found that egg white diluted with water was still germicidal.   Our results show that dilution of alkaline egg white of pH 9.46 with water actually produces solutions which are more alkaline than the original egg white.   The dilution of egg white with water up to 1 part of egg white and 10 parts of water does not produce a medium more suitable for bacterial growth, than the undiluted egg white, in so far as the hydrogen ion concentration is concerned. Laschtschenko (1909) did report a germicidal action toward *Bacillus Zopfii* and *Proteus Zenkeri* in egg white diluted to fifty volumes with nutrient bouillon, but he noted that these two or-

ganisms were more sensitive to the germicidal action of egg white than was *Bacillus subtilis*.   In this case the germicidal action cannot be explained on the basis of the hydroxyl ion concentration.

Laschtschei ko, as was mentioned before, believed that an enzyme of a proteolytic nature was responsible for the germicidal action.   He proved this by heating the egg white to a temperature of 65° to 70°C. for thirty minutes and finding the action destroyed.   That heating under these conditions destroyed the germicidal effect was found to be correct by Horowitz (1902) and Rettger and Sperry (1912), and was also confirmed by our experiments.   Egg white from eggs which had been stored at 30°C. for five days to allow the pH to rise, was beaten together and the pH found to be 9.410.   Half of the egg white was heated to 68°C. for thirty minutes and rapidly cooled.   The pH was again determined and found to be 9.406.   The unheated and heated egg white was then seeded with cultures of *Bacillus subtilis*, *Bacterium coli*, *Serratia marcescens*, and *Pseudomonas pyocyaneus*. Counts were made in each case before and after incubating for six hours at 37°C.   The results of this experiment are found in table 9.

Since heating destroyed the germicidal action, yet did not affect the pH, it was thought that the surface tension might be responsible for the difference in behavior.   A microscopic slide was suspended from the beam of a chainomatic analytical balance so that the end just touched the surface of the liquid in a silica dish supported over the pan on a bridge, and the weight necessary to pull the slide from the surface was determined.   Distilled water was used as a calibrating liquid, its surface tension being taken as 71.78 dynes.   The surface tension determinations were made at 25°C.   The value found for the surface tension of unheated egg white was 52.9 dynes and that for heated egg white 52.2 dynes. The value which we found for the surface tension of egg white is in good agreement with the value (52.69) reported by Quincke (1877).   Apparently the loss of the germicidal action of egg white caused by heat is not due to a change in surface tension.   As heating also precipitates the protein, it was thought that the disappearance of the germicidal action might possibly be due to this

precipitation rather than to the destruction of a proteolytic enzyme. To test this hypothesis it was necessary to produce precipitation by a method which would not destroy an enzyme. Precipitation by ethyl alcohol meets these requirements.

An equal volume of 95 per cent ethyl alcohol was added to fresh egg white and the alcohol removed by evaporation under reduced pressure. The temperature was never raised above 40°C. during the evaporation. The dried egg white was then mixed with water so as to produce a volume of 20 cc. containing about the same total solids as fresh egg white. No bacteria were found on plating the reconstituted egg white. This reconstituted egg white was then inoculated with *Bacillus subtilis*, and plates were poured every two minutes using a loopful to each plate. The plates showed no dimunition in the numbers of bacteria, and a plate made eighteen hours later was extremely thick, showing abundant growth had taken place. The reconstituted egg white had a pH of 9.65.

This experiment indicated that the germicidal action is not due to a proteolytic enzyme. Together with the experiments on heated egg white, it shows however, that hydroxyl ion concentration is not the sole factor which prevents the growth of bacteria.

Scholl (1893) treated egg white with potassium hydroxide and then dialized the solution against 0.75 per cent sodium chloride for twenty-four hour, and tested for the removal of free alkali with indigo calcium disulphonate. He found this solution to be germicidal to *Bacterium typhosum*, and that heating this solution did not destroy the action. He offered as explanation for this, his belief that egg white contained certain atomic groups which possessed a germicidal power. He postulated that heating produced a rearrangement after which the germicidal groups no longer functioned. If the egg white was then treated with alkali (potassium hydroxide) the germicidal groups were supposed to be regenerated and were not again changed by heating. Indigo disulphonic acid changes color at a pH of about 12, so that all of the solutions with which Scholl worked may have had a high pH (even though they were acid to that indicator) and would in

this case have been germicidal, due to their high hydroxyl ion concentration.   Heating, naturally, would not have caused a loss of the action in this case.   These experiments cannot therefore be taken as showing that treatment of heated egg white with alkali and then removing the alkali, produces a germicidal effect in the egg white.

It was found that by dissolving coagulated egg white, formed by heating to 68°C. for thirty minutes, in sodium hydroxide, and then neutralizing with hydrochloric acid until a pH of between 7 and 8 was obtained, the solution possessed no germicidal action, for *Bacillus subtilis* was able to grow.   Thus the germicidal action which Scholl found was probably due to the high hydroxyl ion concentration of his solutions, and not to the rearrangement which he postulated.

It appears from these experiments that there must be some toxic substance present in egg white which is bactericidal (in presence of alkali) and which is either destroyed or rendered inactive by coagulating the albumin by heat or by alcohol.   It was thought that perhaps the surface presented by the coagulated albumin might adsorb the toxic substance, and thus allow the bacteria to grow, and that if such were the case, the addition of a strong absorbtive agent, like activated charcoal, might also adsorb the toxic substance and allow growth.   Experiments of this nature were performed, but the germicidal action was not impaired.

Laschtschenko (1909), and Rettger and Sperry (1912) have shown that *Bacillus subtilis* seems very susceptible to the bactericidal action of egg white.   It will be seen from tables 3 and 7 that in no case did *Bacillus subtilis* grow in egg white, even when the pH was favorable for the growth of other organisms.   Rettger and Sperry found that this organism was frequently killed by the egg white before the first plates could be poured.   Since it appears that *Bacillus subtilis* is unique among those which we studied in its susceptibility, some experiments were undertaken to study this organism and the action of egg white on it.

To determine the rate at which *Bacillus subtilis* was killed, a quantitative attempt was made to plate every two minutes, but

owing to the amount of work which had to be done in the two minute interval it was found to be impossible. A plate poured at the end of six minutes showed that there were no living organisms present, although a seeding of 160,000 per cubic centimeter was made. A cruder qualitative method was then tried, with good results.

The whites from eggs were beaten together and to 20 cc. was added 1 cc. of a *Bacillus subtilis* culture. At two minute intervals a loopful (regular laboratory inoculating loop) was removed and a plate poured from it. By doing the experiment twice, first

TABLE 10

The influence of the age of the Bacillus subtilis culture on the germicidal action of egg white having a pH of 8.38 and 9.24

AGE OF CULTURE	HYDROGEN ION CONCENTRATION	TIME ORGANISM WAS EXPOSED				
		2 minutes	4 minutes	6 minutes	10 minutes	15 minutes
hours	*pH*					
5	8 38	S	S	S	S	S
5	9 24	S	S	S	S	S
8	8 38	S	S	S	S	S
8	9 24	Sp	S	S	S	S
18	8 38	Sp	8C	Sp	S	1C
18	9 24	6C	10C	6C	8C	11C
26	8 38	80C	33C	45C	47C	48C
26	9 24	56C	Sp	31C	45C	43C, Sp

S = sterile plate; C = colonies; Sp = spreader.

using even numbers of minutes and then odd numbers, a plate was poured every minute. The pH of the egg white was 8.20.

One hundred and forty-one colonies appeared after one minute, 89 colonies after two minutes, 17 after three minutes and 6 after four minutes. The plating was continued for one hour, but all of the plates were found to be sterile after exposure to the egg white for five minutes.

Laschtschenko (1909) states that *Bacillus subtilis* was killed as readily in the spore stage as in the vegetative state. His experiment was repeated by us, and it appeared that the spores were more resistant than the vegetative cells; therefore, a more careful

and systematic experiment was undertaken. Cultures of *Bacillus subtilis* of various ages were grown, and using egg white with a pH of 8.38 and 9.24 the same procedure was followed as that described above. The results of this experiment are presented in table 10.

It would appear then that between eight and eighteen hours the organisms became more resistant, and at twenty-six hours had developed so much resistance that the fifteen minutes exposure to the egg white showed almost as many colonies as the two minute interval. A stained microscopic preparation of each culture showed the five- and eight-hour cultures to be all vegetative cells. About 25 per cent of the eighteen hour culture was in the spore stage, while 80 per cent or more of the twenty-six-hour-old culture was spores. From this it would appear that in the case of *Bacillus subtilis* the vegetative cells are subject to the bactericidal action of the egg white, but that the spores are not so readily destroyed, while the pH and age of the egg are not limiting factors.

As there was some question as to the effect of the dilution of egg white on the germicidal action on *Bacillus subtilis*, egg white was diluted with varying amounts of water and with bouillon, up to dilutions of 1:10, and its germicidal effect on this organism determined. The germicidal action was found to persist throughout all the dilutions studied.

Some investigators have shown that the rate of seeding is a factor which must be considered. To show the effect of the rate of seeding of *Bacillus subtilis* on the germicidal action, 20 cc. of fresh egg white was placed in each of five sterile test tubes, various amounts of a culture of *Bacillus subtilis* were added (from 1 to 25 cc.) and the usual qualitative method of testing the germicidal action after ten minutes was followed. No growth occurred on any plates although control cultures made in sterile water at the same time showed upon plating a vast number of colonies.

This shows very strikingly the germicidal action of natural egg white toward *Bacillus subtilis* for after inoculating 20 cc. of egg white of pH 8.2 with 25 cc. of a culture of *Bacillus subtilis* containing about 10,000,000 per cubic centimeter, all organisms were killed in less than ten minutes.

In another attempt to study the toxic substance which gives to egg white its bactericidal action toward *Bacillus subtilis*, the whites of fresh eggs were mixed together and 20 cc. portions were placed in collodion bags, the tops of which were securely closed, and the bags immersed in 20 cc. of sterile distilled water. These

TABLE 11

Fresh egg white was dialyzed in collodian bags for various lengths of time at a temperature of 1 to 5°C. The dialyzed white and the dialyzate were then tested for germicidal action toward *Bacillus subtilis*, exposing the organism to it for ten minutes and then plating out a loopful. A part of the dialyzate was heated to 68°C. for 30 minutes and also tested in the same manner. The pH of the egg white used was 7.87.

TIME DIALYZED	EGG NUMBER	EGG WHITE		DIALYZATE			
				Not heated		Heated	
		pH	Growth	pH	Growth	pH	Growth
hours							
1	1	7 96	—	7 89	+	—	—
1	2	7 92	—	7.89	++	—	—
3	3	7 82	—	8.06	—	7 86	++
3	4	7 92	—	7 89	—	7 89	+++
6	5	7 81	—	7 87	+(?)	7.94	+
6	6	7 88	—	7.84	—	7 90	+++
12	7	7 98	—	7 84	+	7 97	+++
12	8	7.90	—	7 94	+	7.89	+++
24	9	8.00	—	7.89	+	7 97	+++
24	10	7.91	—	7 99	+	7 90	+++
48	11	7.87	—	7 92	++	7 89	+++
48	12	7.90	—	7.64	++	7 69	+++

— = no colonies; + = small number of colonies; ++ = medium number of colonies; +++ = large number of colonies.

were kept for varying lengths of time in a cold room having a temperature between 1° to 5°C.   At the end of the various time periods, duplicate bags were removed and the contents placed in sterile dry test tubes.   The diffusate from each bag was divided into two parts.   One part was heated to 68° to 70°C.

for thirty minutes, the other left unheated. All six tubes were then inoculated with a three hour old culture of *Bacillus subtilis* and a loopful plated out at the end of ten minutes. The hydrogen ion concentration was also determined at the same time. The results are given in table 11, from which we see that in every case the egg white in the collodion bag retained its germicidal action. In the diffusate no germicidal action was detected in the one hour tubes, the rate of diffusion probably being so slow that it was not until the third hour that enough of the toxic substance came through to kill the cells of *Bacillus subtilis*. After six hours of dialysis, growth was again found to take place in the diffusate. It will also be seen that just as egg white itself, when heated to 68° to 70°C. for thirty minutes, was found to lose its germcidal action, so the diffusate when heated produced in every case no diminution in numbers of bacteria. These results were confirmed by repeating the experiment.

In another experiment some fresh egg white was dialyzed for four hours, this length of time producing a diffusate exhibiting the most marked germicidal action. The bags containing the egg white were then removed, and the diffusate was kept at room temperature and its bactericidal action tested at various lengths of time. The diffusate showed germicidal action at the end of two and six hours but at the end of twelve hours slight growth took place and at the end of twenty-four and forty-eight hours distinct growth occurred. These results show that the toxic material which has been separated from egg white by dialysis slowly disappears when the diffusate is kept at room temperature.

Egg white was frozen solid for several days, thawed, and the germicidal action for *Bacillus subtilis* found to be unimpared. Eggs which had been frozen or which had been submerged under water for over two weeks were also found to exhibit the same bactericidal property for this organism. The pH of the white from the frozen and submerged eggs was relatively low and about the same as the egg white of the eggs before giving them these treatments.

The organisms used in this work were obtained from the following sources:

> *Bacterium coli,* stock culture of the laboratory of bacteriology.
> *Bacillus subtilis,* originally obtained from H. J. Conn, Geneva, N. Y.
> *Serratia marcescens,* originally obtained from R. S. Breed, Geneva, N. Y.
> *Bacillus megatherium* and *Bacillus mycoides,* laboratory cultures originally obtained from W. W. Ford, Baltimore, Md.
> *Bacillus cereus, Pseudomonas fluorescens,* and *Proteus vulgaris,* stock culture of the laboratory, originally obtained from the American Type Culture Collection.
> *Pseudomonas pyocyaneus,* isolated in this laboratory from infected egg white by R. Whitaker.

DISCUSSION

Heretofore, the explanation offered to account for the comparative freedom of the normal egg white from bacterial invasion, has been that it possesses germicidal properties. We have shown that this action may be due in part to the hydroxyl ion concentration of the egg white, which rapidly increases from the time the egg is laid until under some conditions a pH of 9.5 may be reached, which does not permit the growth of most bacteria as reported in the literature. Our experiments were designed to show the effect of the hydrogen ion concentration of the egg white on bacterial growth, and do not indicate whether or not bacteria inoculated into egg white of suitable pH multiply as rapidly as they do in broth, bouillon, sugar solutions, etc. In our experiments we have not shown that egg white, at a hydrogen ion concentration at which growth occurred in our standard incubation period of 6 hours, shows no transient germicidal action for shorter periods such as has been demonstrated so clearly for freshly drawn milk by Sherman and Curran (1924). On the other hand, the fact that the bacteria in some instances were not all killed but decreased in numbers during this six hour incubation period does not preclude the possibility that if the incubation were allowed to go for longer periods of time some of the bacteria might survive and might even eventually increase in

numbers. There is also the possibility that bacteria subjected to a hydroxyl ion concentration near the lethal concentration might gain a start on the surface or on the walls of the container above the liquid and gradually neutralize their way and later develop in the whole tube, although all of the bacteria which were completely immersed in the egg white were killed.

From a consideration of the literature on the subject, it seems that the infection of the so-called "fresh" eggs which have been used to determine whether or not bacteria are found in fresh eggs, could occur in any one of three ways; first, the yolk may be infected by infected ovaries before it starts down the oviduct; second, infection may occur as the yolk passes down through the oviduct; and third, infection may be produced after the egg is laid, by contact with wet and soiled nesting material, etc.

The literature is contradictory in regard to the infection of eggs by possibilities one and two, although it does indicate that the yolks of a small percentage of freshly laid eggs are infected. If we consider it possible that the yolk may be infected before it starts down the oviduct, or perhaps in passing down the oviduct, we can see that the hydroxyl ion concentration of the egg white, which is later increased due to the aging of these infected eggs in air for a few days before testing may have a considerable bearing on whether or not bacteria are found in the white. If the yolk is infected before it starts down the oviduct, the organisms will be in a medium which is not germicidal. The yolk does not develop the high hydroxyl ion concentration which limits growth, as is the case with the white. Eggs so infected would, after aging in air for a few days, probably show the presence of organisms in the yolk and not in the white. Mauer (1911), Rettger (1913), Bushnell and Mauer (1914), Hadley and Caldwell (1916), and others, have called attention to the fact that infected eggs usually contain the bacteria in the yolks while the whites are sterile. This has also been found in many instances in our own laboratory.

Laschtschenko (1909) injected *Bacillus subtilis* into the egg. He found that if this organism was injected into the white, it was killed; but if it was injected into the yolk, it grew. He also obtained growth of *Bacillus subtilis* in a mixture of the white and

the yolk but not in the white alone. This organism was killed by a mixture of yolk and white in our experiment. Laschtschenko probably used a strain of *Bacillus subtilis* which was more resistant then the strain which we used.

Rettger and Sperry (1912) found that under anaerobic conditions *Bacillus putrificus* and *Bacillus edematis* grew in egg yolk but were killed in egg white, as shown by appearance of the tubes and by microscopic examination.

If the yolks are sterile as they start down the oviduct, and the oviduct is not sterile, organisms may be present in the white and may reach the yolk in this passage, or before the white develops the high alkalinity after the egg is laid. In this case also it is probable that if the eggs were examined a few days after being laid the yolks would be found to be infected while the whites would be found to be sterile even though the infection actually came from infected white in the oviduct. The investigators of fresh eggs (eggs a few days old) found the organisms chiefly in the yolk and not in the white. If the examinations had been made by a proper method within an hour after the eggs were laid, then it is possible that both white and yolk would have been found to be contaminated. In the light of this discussion it is seen that a reinvestigation of the bacterial content of fresh eggs and the germicidal properties of the secretions in the oviduct would be desirable.

If a sterile egg is actually laid, then infection might occur by the passage of the organisms from the shell to the yolk through the white before the white has had time to develop the high hydroxyl ion concentration which would prevent the organisms from reaching the yolk later. At room temperatures the organisms must pass through the shell, and shell membranes, and the white, in a period of not more than three or four days, in order to infect the yolk. Whether such rapid passage is possible is rather doubtful. Progress through these structures must necessarily be slow. It should also be remembered that the yolk of the fresh egg is surrounded with an egg white gel, the viscosity of which would tend to retard the penetration of the bacteria.

Some of the investigators of the question as to whether or not

eggs are contaminated with bacteria when laid, apparently did not believe it possible that bacteria could penetrate into the egg within the first few days after being laid, when stored under fairly dry conditions, for they frequently did not examine them for the presence of bacteria until several days after the eggs were laid. If it is impossible for the bacteria to penetrate the shell, the membranes, and the thin and thick egg white within a few days, then the only explanation for the presence of bacteria in the yolk of the so-called fresh eggs is that they were actually present when the eggs were laid.

We have pointed out the importance of the hydroxyl ion concentration of the white in resisting bacterial invasion. We now must account for the spoilage of eggs and decomposition of the white due to bacterial growth. Some points in this discussion may also apply to the invasion by molds but we are considering in this paper mainly invasion by bacteria. Many factors may play a part in regulating this invasion and some of them will be considered in a later paper, but in so far as the hydroxyl ion concentration is concerned we may mention a few possibilities which seem to fit in with our present knowledge.

If eggs with infected yolks are kept at a temperature which permits bacterial growth, the organisms may increase in numbers and gradually work their way out into the white neutralizing their way with their metabolic products as they go.

If a sterile egg is laid, it may be infected with bacteria in several ways. If moisture condenses on the shell, or if moist nesting material adheres to the shell for some time and the temperature is suitable, bacteria will slowly invade the egg. The organisms probably first develop in the shell membranes where they are not exposed to the high alkalinity of the white and then gradually invade the white as their metabolic products reduce its alkalinity. It has been found that egg white which is highly infected with organisms is usually considerably more acid than the uninfected egg white, the pH frequently dropping to near the neutral point.

Another point of invasion of bacteria into eggs is by way of the air space. It is probable that bacteria can grow on the membrane forming the surface of the air space without very many of

them coming in contact with the alkaline albumin. The alkalinity of the egg white would be gradually neutralized by their metabolic products, as mentioned above, thus permitting the organisms to penetrate further and further into the white. Candling evidence seems to bear out this idea.

Stored eggs are never turned, and the yolks may move through the white until they touch the shell. Bacteria then are able to grow where this contact is made, entering from the shell and its membranes and growing in the yolk. As was mentioned before, acid by-products, liberated by bacterial growth, gradually neutralize the alkalinity of the white surrounding this point of contact. Candling evidence also seems to bear out this idea.

This study has all been carried out on a relatively few organisms, but as egg white which had a hydroxyl ion concentration corresponding to pH 9.5 was germicidal to all of them, we have concluded that the hydroxyl ion concentration is the most important variable factor in limiting the growth of organisms in natural egg white. We have not eliminated the possibility of there being organisms, or even strains of the organisms which we have investigated, that can withstand this high alkalinity of the natural egg white, but such organisms are probably not very numerous.

There is one point which we have not taken into consideration in this work, that is the variation in the germicidal action of egg white of various pH values with change in temperature. All of our tests of the germicidal action were made by inoculating egg white at room temperature and then placing the tubes in an air thermostat at 37°C. and determining the count at the end of six hours. One would expect that the death rate would be slower at the lower temperatures, but that the death of the bacteria would still occur.

Rettger and Sperry (1912) state "The last two series of results seem to indicate that the bactericidal action of the egg-white is accelerated with increased temperature."

It is readily seen from the investigation reported here that the hydroxyl ion concentration of the raw egg white may assume a very important rôle in regard to the storage of eggs. It is also

apparent that a marked difference exists between the effect of the hydroxyl ion on the germicidal action of raw and heated egg white. The bacteria are able to grow in egg white which has been heated, even though it has a hydroxyl ion concentration which would limit growth in the unheated material. There is apparently some other factor in raw egg white, in addition to the hydroxyl ion concentration, which affects the growth of bacteria. This other factor does not, however, minimize the practical importance of the hydroxyl ion concentration in regulating the growth of bacteria in raw egg white.

SUMMARY

1. The so-called germicidal action of raw egg white is markedly influenced by the hydroxyl ion concentration, which increases rapidly during the first few days of storage of untreated eggs in a ventilated room. It was found that the hydroxyl ion concentration of the white corresponding to that at the time the egg was laid would permit growth, while the hydroxyl ion concentration corresponding to the whites of eggs aged a few days in air was germicidal to the following organisms: *Bacterium coli*, *Pseudomonas pyocyaneus*, *Serratia marcescens*, *Proteus vulgaris*, *Pseudomonas fluorescens*, *Bacillus cereus*, *Bacillus megatherium*, and *Bacillus mycoides*.

2. The conflicting results of previous workers may be partially explained on the basis that they did not take into account the age of the egg and, consequently, the hydroxyl ion concentration, when testing for the germicidal action of the egg white.

3. For vegetative cells of the strain of *Bacillus subtilis* which we used, normal egg white at all hydrogen ion concentrations was decidedly bactericidal, while the spores were more resistant.

4. Heat and alcohol coagulation of the egg white destroyed its germicidal action on the vegetative cells of *Bacillus subtilis*.

5. The toxic substance which kills the vegetative cells of *Bacillus subtilis* can be separated from egg white by dialysis, the diffusate gradually becoming non-germicidal on standing.

REFERENCES

AVERY, O. T., AND CULLEN, G. E.   1919   Jour. Exper. Med., **30**, 359.

BAINBRIDGE, F. A.   1911   Jour. Hyg. (Cambridge), **11**, 341.

BUNKER, J. W. M.   1917   Abs. Bacteriol., **1**, 31.

BUSHNELL, L. D., AND MAUER, O.   1914   Kan. Agr. Exper. Sta. Bul. 201.

COHEN, B., AND CLARK, W. M.   1919   Jour. Bacteriol., **4**, 409.

HADLEY, P B , AND CALDWELL, D. W.   1916   R. I. Agr. Exper. Sta. Bul. 164.

HEALY, D. J., AND PETER, A. M.   1925   Amer. Jour. Physiol., **74**, 363.

HOROWITZ, A.   1902   Thesis, Paris, Abs. in Baumgarten's Jahresber., **19**, 984.
        (Original not seen.)

ITANO, A.   1916   Mass. Agr. Exper. Sta. Bul. 167.

LASCHTSCHENKO, P.   1909   Ztschr. Hyg., **64**, 419.

McCLENDON, J. F., AND MEDES, G.   1925   Physical Chemistry in Biology and
        Medicine, Philadelphia, Pa., 205.

MAUER, O.   1911   Kan. Agr. Exper. Sta. Bul. 180.

PARASCANDOLE, C.   1893   Centbl. Bakt., **14**, 291.

QUINCKE   1877   As given by LANDOLT, H., BÖRNSTEIN, R., AND ROTH, W. A.:
        Physikalisch-Chemische Tabellen, Berlin, 4th edition, 121 (1912).

RETTGER, L. F.   1913   Storrs, Conn. Agr. Exper. Sta. Bul. 75.

RETTGER, L. F., AND SPERRY, J. A.   1912   Jour. Med. Research, **26**, 55.

SCHOENHOLZ, P., AND MYER, K. F.   1921   Jour. Inf. Dis., **28**, 384.

SCHOLL, H.   1893   Arch. für Hyg., **17**, 535.

SHERMAN, J. M., AND CURRAN, H. R.   1924   Proc. Soc. Exper. Biol. and Med.,
        22, 15.

SPERRY, J. A.   1913   Science, N.S., **38**, 413.

SPERRY, J. A., AND RETTGER, L. F.   1915   Jour. Biol. Chem., **20**, 445.

TICE, W. G.   1911   Thirty-fifth Ann. Rept. State Bd. Health, N. J., 275.

TURRÓ, R.   1902   Centbl. Bakt., Abt. 1, Orig., **32**, 105.

WURTZ, R.   1890   Semaine Med , Paris, **3**, 21.

GENTIAN VIOLET LACTOSE PEPTON BILE FOR THE DETECTION OF B. COLI[1] IN MILK

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Since the original researches of Churchman (1912) on the selective action of the triphenylmethane dyes on bacteria the use of these dyes in bacteriological media has become quite extensive. Their use in media for the detection of *B. coli* promptly suggested itself. Hall and Ellefson (1918) recommended the use of gentian violet in the lactose broth used for the presumptive test for *B. coli* in water, and published a note (1918a) on the use of the same in demonstrating *B. coli* in milk. Muer and Harris (1920) showed the value of brilliant green lactose pepton bile in the elimination of presumptive tests due to anaerobes and stated that 10 per cent or more of gas in the medium is an indication of the presence of *B. coli* which needs no confirmation. Winslow and Dolloff (1922) demonstrated the effect of bile in reducing the inhibiting action of gentian violet and brilliant green for members of the *B. coli* group.

The medium which we will describe here for detecting *B. coli* in milk was formulated after a consideration of the above articles and as a result of our own experience with plain lactose broth, gentian violet lactose broth, and gentian violet lactose pepton bile.

Our interest in a reliable method for detecting *B. coli* in milk was created by our desire to include in a bacteriological study of the milk supply of the city[2] an estimation of the *B. coli* content.

[1] The term *B. coli* is used in this paper to refer to all members of the group: i.e., all Gram-negative, non-spore-forming bacilli which ferment lactose with the formation of gas and grow aerobically on standard solid media.

[2] The details of this work have not been published as yet but an abstract of a preliminary paper will be found in the American Journal of Hygiene, May, 1925.

We discarded plate methods for this purpose, not because these methods are altogether unreliable, but because it has come to be considered essential to demonstrate that an organism produces gas from lactose before it can be considered as belonging to the *B. coli* group.

Plain lactose broth as recommended for water analysis was tried and discarded as unreliable because of the frequent occurrence of positive presumptive tests in the higher dilution while the low dilutions were negative. We were at a loss to explain these results except on the ground that when acid producing organisms other than *B. coli* are present in great numbers in the inoculum they promptly develop enough acid to inhibit the growth of *B. coli*. Since the majority of the non-coliform organisms in milk are Gram-positive, the above line of reasoning suggested the use of gentian violet in the broth.

That our assumption is probably correct was shown in the comparison of results obtained in plain lactose broth and lactose broth to which gentian violet in concentration of 1–100,000 was added. The results not only indicated that the addition of gentian violet to lactose broth improves the medium for use in milk work but also that gentian violet even in the low concentration had a tendency to inhibit colon organisms.

Since Winslow and Dolloff (1922) had demonstrated that bile incorporated in the medium reduced the inhibiting effect of gentian violet for members of the *B. coli* group, we decided to try medium containing bile. The medium[3] used was the same as described by Muer and Harris (1920) except that we used gentian violet in a concentration of 1–25,000 instead of brilliant green and adjusted the reaction to pH 7.8 before adding the dye.

Nineteen samples of pasteurized milk and 23 samples of raw milk were examined for *B. coli* with this medium and with plain

[3] "Heat 1 liter of distilled water in a double boiler until water in outer vessel, boils. Add 50 grams of bile and 10 grams of pepton, stirring until all ingredients are dissolved; continue boiling for one hour. Remove from flame and add 10 grams of powdered lactose. Filter through cotton flannel until clear. To each liter of filtrate add 10 cc. of a 1 per cent solution of brilliant green. Tube and autoclave for fifteen minutes at 15 pounds pressure."

lactose broth. Dilutions from 1 cc. to 1/100,000 cc. were made on all samples and incubated at 37°C. Of the 19 pasteurized samples the lactose broth showed a total of 8 positive both in twenty-four and forty-eight hours, against 10 in twenty-four hours and 14 in forty-eight hours in gentian violet lactose bile. Of the 23 raw milk samples tested, the lactose broth gave 51 positives at the end of twenty-four hours and 54 in forty-eight hours, as compared with a total of 80 in twenty-four hours and 81 in forty-eight hours with the gentian violet medium.

The results indicate that the gentian violet lactose pepton bile is far superior to plain lactose broth for the detection of lactose fermentation in milk and gives no such indication of inhibition of these organisms in the higher dilution as was indicated in the case of plain gentian violet lactose broth.

In none of the tests so far described were confirmatory tests made for the presence of organisms of the *B. coli* group in the tube showing gas. The gentian violet lactose pepton bile medium was adopted for use in estimating the *B. coli* content in the milk supply. For this purpose we used decimal dilutions and in order to confirm our analyses with respect to *B. coli* we selected in each case the tube from the highest dilution which showed gas and plated it on eosin-methylene-blue agar for the observation of typical colonies on this medium. This is essentially the partially confirmed test as described in the Standard Method for Water Analysis.

One thousand and ten (1010) such determinations were made, and all except ten (10) showed typical growth for members of the *B. coli* group. The ten tubes which did not confirm by this method were not examined further but were considered as negative for *B. coli*. It is possible that some or all of these contained *B. coli* but the growth was atypical or the typical appearance was obscured by other organisms. However, if we consider these as false tests we still have more than 99 per cent of the tubes examined showing good evidence that the gas produced was due to the activity of some member of the *B. coli* group, and we believe that for all practical purposes it may be considered that gas formation in gentian violet lactose pepton bile which has been

TABLE 1

Showing the number of positive tests obtained in each quantity of milk tested for 5 different media

Raw milk

MEDIA	1 cc			1-10 cc			1-100 cc			1-1000 cc			1-10,000 cc			1-100,000 cc			TOTAL NUMBER POSITIVE, ALL QUANTITIES	
	Number of samples examined	Number of positives—24 hours	Number of positives—48 hours	Number of samples examined	Number of positives—24 hours	Number of positives—48 hours	Number of samples examined	Number of positives—24 hours	Number of positives—48 hours	Number of samples examined	Number of positives—24 hours	Number of positives—48 hours	Number of samples examined	Number of positives—24 hours	Number of positives—48 hours	Number of samples examined	Number of positives—24 hours	Number of positives—48 hours	24 hours	48 hours
Plain lactose broth	60	23	29	60	32	36	60	29	31	60	13	18	60	4	8	24	0	3	101	125
G. V. L. P. Bile, 5 grams*	60	39	56	60	32	47	60	22	37	60	10	22	60	3	7	24	0	0	106	171
G. V. L. P. Bile, 10 grams	60	46	53	60	39	45	60	26	40	60	13	28	60	2	7	24	0	1	126	174
G. V. L. P. Bile, 25 grams	60	45	52	60	39	47	60	24	32	60	14	24	60	4	11	24	0	0	126	166
G. V. L. P. Bile, 30 grams	60	50	54	60	31	46	60	26	41	60	11	27	60	3	13	24	1	3	125	184

Average percentages of gas†

MEDIA	1 cc		1-10 cc		1-100 cc		1-1000 cc		1-10,000 cc		1-100,000 cc		TOTAL	
	24 hours	48 hours	24 hours	48 hours	24 hours	48 hours	24 hours	48 hours	24 hours	48 hours	24 hours	48 hours	24 hours	48 hours
Plain lactose broth	8 1	10 7	12 6	16 9	13 8	19 5	16 9	21 6	11 2	17 5	16 9	13 6	16 9	8
G. V. L. P. Bile, 5 grams	16 3	14 7	16 3	45 4	15 0	42 9	17 5	37 3	13 3	29 3	—	16 1	43 8	8
G. V. L. P. Bile, 10 grams	24 1	53 6	21 4	57 1	19 5	49 5	16 9	43 3	32 5	42 9	65	21 8	51 4	
G. V. L. P. Bile, 25 grams	32 3	78 6	29 6	66 6	25 3	57 0	17 1	54 0	12 5	53 2	—	27 5	61 3	
G. V. L. P. Bile, 30 grams	35 2	39 5	27 3	65 3	18 3	55 6	31 8	49 3	15 0	45 4	20	23 7	60 0	

* Formula for G. V. L. P. Bile = 5 grams peptone, 10 grams lactose, gentian violet (Coleman and Bell) 1-2500, bile as indicated.

† Average percentages of gas in tubes positive in above table

TABLE 2

Showing the number of positive tests obtained in each quantity of milk tested for 5 different media

Pasteurized milk

MEDIA	1 cc — samples examined	1 cc — positives 24 hours	1 cc — positives 48 hours	1/10 cc — samples examined	1/10 cc — positives 24 hours	1/10 cc — positives 48 hours	1/100 cc — samples examined	1/100 cc — positives 24 hours	1/100 cc — positives 48 hours	1/1000 cc — samples examined	1/1000 cc — positives 24 hours	1/1000 cc — positives 48 hours	1/10,000 cc — samples examined	1/10,000 cc — positives 24 hours	1/10,000 cc — positives 48 hours	TOTAL, ALL DILUTIONS — 24 hours	TOTAL, ALL DILUTIONS — 48 hours
Plain lactose broth	40	2	4	40	0	0	40	0	0	40	0	0	40	0	0	2	4
G. V. L. P. Bile, 5 grams	40	1	9	40	0	2	40	0	0	40	0	0	40	0	0	1	11
G. V. L. P. Bile, 10 grams	40	3	8	40	0	2	40	0	0	40	0	0	40	0	0	3	10
G. V. L. P. Bile, 25 grams	40	4	6	40	0	3	40	0	1	40	0	0	40	0	0	4	10
G. V. L. P. Bile, 50 grams	40	3	10	40	1	1	40	0	0	40	0	0	40	0	0	4	11

Average percentages of gas

MEDIA	1 cc (24 hr)	1 cc (48 hr)	1/10 cc	1/100 cc
Plain lactose broth	40	25	—	—
G. V. L. P. Bile, 5 grams	10	33	27	—
G. V. L. P. Bile, 10 grams	22	39	43	—
G. V. L. P. Bile, 25 grams	29	48	43	10
G. V. L. P. Bile, 50 grams	33	52	80	—

inoculated with milk or diluted milk is a positive indication of the presence of organisms of the *B. coli* group.

Since the medium as described above is quite expensive because of the high content of bile, we decided to run some comparisons with a medium in which lower percentages of bile were present. We made up four batches of the medium containing 5, 10, 25, and 50 grams of bile per liter respectively. These together with plain lactose broth were tested on 60 samples of raw milk and 40 samples of pasteurized milk. The results are tabulated in tables 1 and 2.

It will be noted that in raw milk the medium containing 5 per cent of bile shows a slight superiority over those containing less bile, but in pasteurized milk there is practically no difference. It will also be noted that the total number of positive tests obtained from the raw samples with the medium containing 2.5 per cent of bile was less than the total number obtained with either the 1.0 per cent or the 0.5 per cent bile medium. In other words, the curve from 0.5 per cent bile to 5 per cent bile is not a smooth one. We feel that the superiority indicated for the 5 per cent medium is not to be considered too seriously. The possibilities are that the figures are the result of chance, and if a larger number of comparisons was made the curve would smooth out into a straight line at least with respect to the 1, 2.5, and 5 per cent media. However, the average percentage of gas, which perhaps may be considered as indicating more favorable conditions for growth, is also higher for the 5 per cent and 2.5 per cent bile media than for the lower percentage bile media. The difference between the 2 per cent and the 1 per cent medium is not so great as to indicate a decided superiority. We therefore feel that the 1 per cent medium can be used in routine work with the assurance that *B. coli* will be detected just as surely as with a medium containing more bile. Using this concentration of bile the cost of materials is less than one-fourth of that for the 5 per cent medium and the medium becomes a practical one for use in routine work.

SUMMARY

Plain lactose broth, gentian violet lactose broth, and gentian violet lactose pepton bile have been compared as to their reliability for detecting *B. coli* in milk. It was found that gentian violet lactose pepton bile is the most reliable of these media for this purpose, and the formation of gas in this medium when inoculated with milk or diluted milk is a positive indication of the presence of *B. coli* which for practical purposes needs no confirmation.

REFERENCES

CHURCHMAN, JOHN W. 1912 The selective bactericidal action of gentian violet. Jour. Exper. Med , **16**, 221.

HALL, I. C., AND ELLEFSON, L. J. 1918 The elimination of spurious presumptive tests for *B. coli* in water by the use of gentian violet. Jour. Bacteriol., **3**, 329.

HALL, I. C., AND ELLEFSON, L. J. 1918a A brief note of the use of gentian violet in presumptive tests for *B coli* in milk with reference to sporulating anaerobes. Jour. Bacteriol., **3**, 355.

MUER, T. C., AND HARRIS, R. L. 1920 The value of brilliant green in eliminating errors due to anaerobes in the presumptive test for *B. coli*. Amer. Jour. Pub. Health, **10**, 874.

WINSLOW, C.-E. A., AND DOLLOFF, A F. 1922 Relative effect of certain triphenylmethane dyes upon growth of bacilli of the colon group in lactose broth and lactose bile. Jour. Inf. Dis , **31**, 302.

THE FATE OF B. COLI AND B. AEROGENES IN SEWAGE PURIFICATION[1]

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INTRODUCTION

One of the outstanding purposes of sewage treatment is to obtain a substantial reduction in the number of bacteria present. That the intestinal bacteria which constitute the bulk of the organisms in the incoming sewage are reduced considerably as a result of the treatment has long been known; but the factors influencing this reduction from the standpoint of practical operation of a sewage disposal plant have not been fully studied. Further it is conceivable and probable that even though these intestinal organisms are reduced in numbers, they may play a temporary and yet an important rôle in the process of purification itself. The following work was undertaken as an attempt to clarify some of these factors which operate in the reduction of *B. coli* and *B. aerogenes* in a sewage disposal plant and to determine their fate, and rôle.

The work will be reported in two parts, In the first part will be given the results of a survey of the distribution of these organisms in the different units of a sewage disposal plant. In the second part laboratory experiments will be reported on the fate of *B. coli* and *B. aerogenes* in fresh solids and in sludge to which a daily addition of fresh solids was made.

Method

The numbers of $B.\overline{}coli$ and *B. acrogenes* were determined by the dilution method. One cubic centimeter portions of the various dilutions of the samples were inoculated into gentian violet lactose tubes. After forty-eight hours incubation at 37°C. these were streaked on the surface of solid gentian violet-eosin-methylene-bile agar. This is the ordinary standard agar used for this purpose as modified by Skinner and Murray (1924) with the addition of gentian violet at a concentration of 1:100,000 to repress the development of spreading colonies.

PART I

The numbers of *B. coli* and *B. aerogenes* were determined by the above method for a period of seven months in the different units of the sewage disposal plant at Plainfield. The sewage at this plant after passing through the Riensch-Wurl screen is treated in a series of Imhoff tanks, the first of which is used as a preliminary sedimentation tank. The effluent of the tanks is sprinkled intermittently over trickling filters. The effluent of the filter bed is further passed through a final settling tank.

Beginning with the middle of August 1925 up to the end of March 1926 samples were taken on Wednesday at 11 o'clock at which time the concentration of the sewage can be taken as an average for the rest of the day. Twenty minutes after the sampling of the screened influent a sample was taken from the effluent of tank 1 which was being used as a preliminary sedimentation tank. A sample of the sewage after going through the remainder of Imhoff tanks was collected forty minutes after. Half an hour was allowed before taking the next sample from the filter bed effluent and ten minutes after this, the effluent from the secondary sedimentation tank was collected. These intervals represent the average flow time through the different units. By this means comparative studies could be made as to the effect of the different units upon *B. coli* and *B. aerogenes* of what was initially the same sewage.

TABLE 1

The numbers of B. coli and B. aerogenes in the different units of a sewage disposal plant

	INFLUENT		TANK 1 EFFLUENT		UNIT EFFLUENT		FILTER EFFLUENT		FINAL EFFLUENT	
	Coli	Aerogenes	Coli	Aerogenes	Coli	Aerogenes	Coli	Aerogenes	Coli	Aerogenes
	thousands	thousands	thousands	thousands	thousands	thousands	thousands	thousands	thousands	thousands
August 12 ..	1,000	1,000			100	10	1	1	100	10
August 19 ...	1,000	1,000	100	10	50	10	1	5	5	10
September 2	500	500	10	50	50	10	10	1	1	1
September 9 .	100	100	500	500	500	100	10	10	10	10
September 23	100	100	500	500	100	1,000	100	100	50	10
September 30	100	100	50	50	10	10	10	1	50	1
October 7 .	100	100	50	10	100	100	1	1	1	1
October 14	100	100	50	10	100	100	1	1	5	5
October 21 .	50	50	10	50	100	50	5	5	5	5
October 28	10	10	10	50	10	5	10	10	1	1
November 4	10	10	10	10	10	10	5	5	5	1
November 18 .	100	5	500	500	10	5	1	0 1	10	10
November 25	500	10	10	10	1	5	10	10	10	10
December 2 .	10	10	100	100	10	10	1	0 1	1	0 1
December 16 .	100	100	1	1	5	5	1	1	1	1
December 23 ..	5	1	1	1	1	1	0 1	0 1	1	1
December 30	10	10	10	10	10	10	10	10	10	0 1
January 6	10	10	10	10	50	50	1	0 1	5	1
January 13 ..	1	1	10	10	50	50	1	1	5	5
January 20 .	50	50	50	50	100	100	1	1	0 1	0 1
January 27	5	5	5	5	1	1	0 1	0 1	1	1
February 3 .. .	10	5	100	1	50	5	1	0 1	1	0 1
February 19 . .	5	5	5	5	5	10	5	1	10	10
February 24	5	5	5	5	10	10	5	5	1	5
March 3	5	5	10	1	5	5	1	1	10	1
March 10	5	5			10	10	0 1	0 1	5	5
March 17	5	1			100	50	1	1	1	0 1
March 24..	1	1			1	10	5	5	0 1	0 1
March 31.	50	50			5	5	1	1	1	0 1

Discussion of results

The results are given in table 1 from an examination of which it can be seen that the fluctuations are great and that the results for *B. coli* and *B. aerogenes* follow each other quite closely.   Hence

the results for these two organisms were added and monthly averages made. These figures are given in tables 1 and 2, which contain also the monthly average of suspended solids in the units. As far as seasonal correlation is concerned it can be said in a general way that the numbers of *B. coli* and *B. aerogenes* in the incoming sewage were lower in winter months than in the fall, but the reduction in numbers due to treatment was not necessarily higher in the winter months. In fact in January and February the tank effluent showed a decided increase over the original numbers (see fig. 1).

TABLE 2

Monthly averages of B. coli and B. aerogenes and of suspended solids in the different units of the plant

	INFLUENT		TANK 1 EFFLUENT		UNIT EFFLUENT		BED EFFLUENT		FINAL EFFLUENT	
	Solids*	Bacteria†	Solids	Bacteria†	Solids	Bacteria†	Solids	Bacteria†	Solids	Bacteria†
	p p m	thousands	p p m	thousands	p p m	thousands	p p m	thousands	p p m	thousands
August .. .	251	2,000		110	62	85	32	1 7	25	62
September .	189	400		540	59	445	82	60	46 4	33
October .	231	130		60	45	141	28	8	39 0	6
November .	186	212		347	63	14	44	10	25	15
December ..	189	61		56	64	13	67	6	43	3 3
January.. .	229	34		37	81	100	65	1 3	44 8	4 5
February .	164	12		40	88 8	30	50 4	5 7	47 4	9 0
March .	186	25		11	82	40	32	3 2	23	4 7

* The figures on solids were obtained by the courtesy of Mr. Downes.
† Bacteria *B. coli* and *B. aerogenes*.

Table 3 gives the results calculated in terms of percentage reduction as compared with the incoming sewage for solids and for bacteria. On examining this table it can be seen that the greatest reduction in the bacteria took place after the sewage had passed through the filter beds. In the tanks the reduction was either inappreciable or altogether absent; in fact on a number of occasions there was a decided increase in the number of *B. coli* and *B. aerogenes*. The increase was most pronounced in January and February. An explanation of this will be offered below. With only one exception, once the sewage passed over
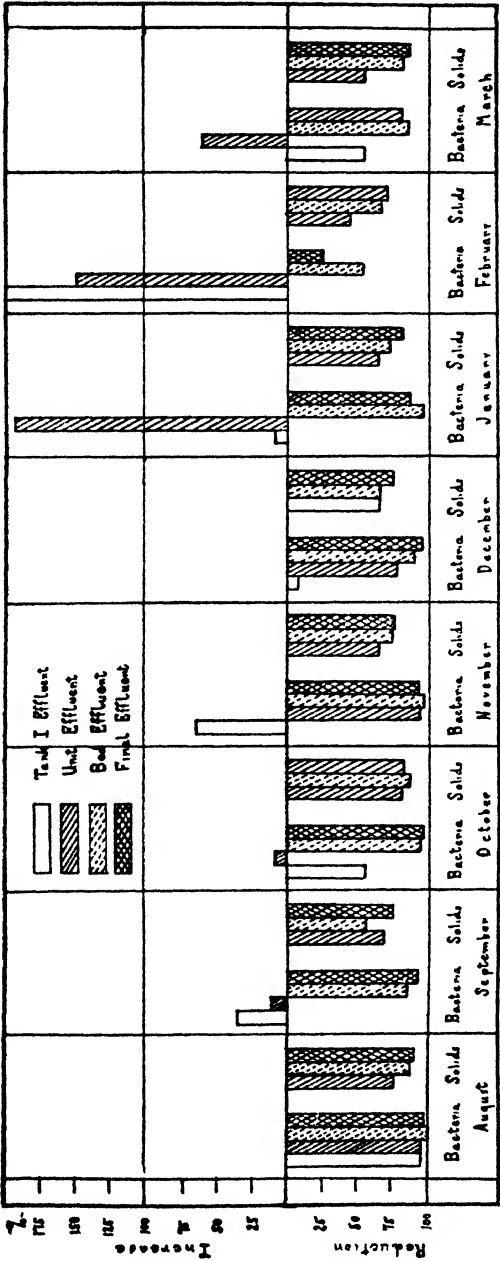
FIG. 1. MONTHLY AVERAGES OF THE REDUCTION OR INCREASE OF B. COLI AND B. AEROGENES AND OF SUSPENDED SOLIDS IN THE DIFFERENT UNITS OF A SEWAGE DISPOSAL PLANT

the beds, a very uniform reduction took place which averaged for the whole period about 89 per cent of the original numbers. The only apparent exception to this seems to be in February when only a 53 per cent reduction took place in the beds. However, on referring to the table it will be seen that the numbers of *B. coli* and *B. acrogenes* that went through the beds was low and that the percentage reduction was also low because the incoming solids in the month had low figures.

TABLE 3

Reduction of B. coli and B. aerogenes and of solids in the different units of the plant

	REDUCTION IN SETTLING TANK		REDUCTION IN IMHOFF TANKS		REDUCTION IN BEDS		REDUCTION IN FINAL TANK	
	Solids	Bacteria	Solids	Bac-teria	Solids	Bac-teria	Solids	Bac-teria
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
August . . .		−95	−75	−96	−88	−100	−90	−97
September		+35	−69	+11	−57	−85	−76	−92
October. . . .		−54	−81	+9	−88	−94	−83	−96
November		+63	−66	−94	−76	−96	−87	−93
December . .		−8	−66	−79	−65	−90	−77	−95
January. . .		+9	−65	+194	−72	−96	−81	−87
February.		+230	−46	+150	−69	−53	−71	−25
March		−56	−56	+60	−83	−87	−88	−81
Average.		+15.5	−65	+190	−75	−89	−82	−83

* + is increase; − is decrease.

As far as reduction of the number of *B. coli* and *B. aerogenes* is concerned there is nothing to be gained from the final settling tank in fact there is usually a slight increase in the bacterial numbers due to this final treatment.

The suspended solids on the other hand were removed effectively in the tanks, an average of 65 per cent reduction of the original taking place for the whole period. The solids were reduced in the beds an additional 10 per cent. In the final settling tanks a further removal of 7 per cent over bed effluent took place. It thus becomes apparent that the reduction of the bacteria is not directly correlated with the reduction of solids. In the tanks where reduction of solids is most pronounced a

bacterial reduction might either be small or there might even be an increase. On the other hand with a 10 per cent reduction of solids in the beds we get 89 per cent reduction of the bacteria. Furthermore while the solids are reduced further in the final settling tank there is no corresponding decrease in the bacteria. All these facts seem to indicate that the bacteria are not associated with the solid particles to a great extent. The determinations of solids were made by passing the material through Gooch crucibles. This would include all suspended solids except colloidal particles and dissolved solids. It would therefore seem

TABLE 4

Relation of fineness of particles to the distribution of bacteria

	SOLIDS	BACTERIA PER CUBIC CENTIMETER	INCREASE OR DECREASE OF SOLIDS OVER ORIGINAL	INCREASE OR DECREASE OF BACTERIA OVER ORIGINAL	BACTERIA PER GRAM DRY SOLIDS
	per cent	*millions*	*per cent*	*per cent*	*millions*
1 shaken	2 40	34 0			1,417
2 centrifuged a minute supernatant liquid	0 72	15 9	−70 0	−53 2	2,208
3 centrifuged 1 minute deposit	10 92	14 6	+355	−57 0	134
4 centrifuged 10 minute supernatant liquid	0 61	6 2	−74 6	−81 8	1,016
5 centrifuged 10 minute deposit	15 35	244 0	+539 6	+618 0	1,589
6 centrifuged 10 minute supernatant liquid filtered	0 52	1 2	−78 3	−96 4	234

that the bacteria are associated mainly with the fine colloidal particles.

The following experiments demonstrate that the bacteria are actually associated with the fine particles. Liquid from a tank was taken and different portions centrifuged for one to ten minutes and one portion filtered after centrifuging. Solids and the total number of bacteria were determined from the supernatant liquid and the deposit. The results as given in table 4 show that on centrifuging for one minute the percentage reduction of solids in the liquid was greater than that of bacteria. On centrifuging for ten minutes the reduction of bacteria in the

liquid was greater than that of the solids. On the other hand the solids in the deposit on centrifuging for one minute showed a percentage increase while the bacteria decreased; and on centrifuging for ten minutes the increase in both cases was of the same order. In the filtered and centrifuged liquid, reduction in bacteria was of a greater order than that of solids. The bacteria per gram of dry solids were highest in the supernatant liquid of the one minute centrifuged material. They decreased on prolonged centrifuging. The bacterial content per gram of dry solids in the deposit of 1 minute centrifuged material was low and increased on prolonged centrifuging. All these facts would indicate that the bacteria are mostly associated with the finer particles in sewage.

In view of this conclusion an explanation for the actual increase in the numbers of *B. coli* and *B. aerogenes* in passing through the Imhoff tanks in the winter months can be offered. That it is not correlated with a corresponding increase in total solids is clear. To assume an actual multiplication of the organisms in the tanks during these months is untenable. On the other hand it is evident that rapid digestion does not take place in these months. Hence, solids are being added constantly to the digestion chamber of the tanks which are not being digested. These are accumulating constantly and naturally displacing the liquid. It is in this displaced liquid from the tanks that we have to look for an explanation of increased numbers of *B. coli* and *B. aerogenes* in the tank effluents. This liquid has a relatively high percentage of colloidal material and hence more bacteria. Furthermore lower temperatures in winter retard the rate of decrease of these organisms hence more of them are found in the displaced liquid than would be the case in summer months. This last statement is borne out by the temperature experiment in which the *B. coli* were observed to live over a longer period of time at lower temperatures than at high temperatures. This phase of the problem will be fully discussed in a later publication. Thus, in the winter months when digestion is slow not only is more of the liquid from the tanks displaced due to piling up of solids but the number of the intestinal organisms surviving in the tanks is greater due to

the reduction in acid production by these organisms which is the dominating factor in reducing their number.

In view of the above conclusion, that the bacteria are mostly associated with the finer colloidal particles of sewage, it would seem to be a feasible plan to attempt to reduce their numbers by adding a coagulating agent to flock out these colloidal particles. The points to be considered in such a scheme would be: (1) The selection of a practical and efficient coagulating agent. (2) The addition of this material in the necessary concentrations to sewage which has undergone a preliminary sedimentation in order to remove most of the settable solids and thus reduce the concentration of the coagulant needed. (3) The detention period necessary for the settling of the coagulated material.

It would seem that such a scheme could be used in any one of the following ways:

1. Where no trickling filters or sand beds follow sedimentation and digestion tanks.

2. In addition to trickling filters where very pure effluents are required.

3. In combination with chlorination thereby reducing the amounts of chlorine required.

4. Instead of chlorination.

Such a procedure would have an advantage over chlorination in the fact that the colloidal and putrescible material in the sewage would be settled out and retained in the tanks, while in chlorinated sewage such substances decompose in the stream.

Summary and conclusions

A study was made over a period of seven months on the distribution of *B. coli* and *B. aerogenes* in the different units of a sewage disposal plant.

There were no significant differences between the relative numbers of *B. coli* and *B. aerogenes* at a given date for a given unit.

The weekly fluctuations were great but monthly averages show that the numbers in the incoming sewage were lower in the winter months.

Ordinarily in passing through the Imhoff tanks a material

reduction of the numbers of *B. coli* and *B. aerogenes* does not take place.

A reliable and material reduction of these organisms invariably takes place in the filter beds, averaging to 89 per cent of the original numbers.

In the final settling tanks there is no further reduction, but sometimes an increase.

There is no correlation between solid removal and bacterial reduction in the different units. Whereas maximum removal of solids takes place in the tanks, maximum reduction of *B. coli* and *B. aerogenes* takes place in the beds, with no corresponding decrease in solids.

Bacteria are associated with the fine suspended particles which pass through the tanks and their numbers are reduced in the beds due to unfavorable conditions for continued viability.

There is an increase in the number of these organisms in the effluent from the tanks as compared with the incoming sewage in winter months due to more suspended solids passing out of the tanks. The lower temperature causes a low rate of digestion in the tank which induces (1) more of the liquid in the tanks to be displaced into the flow compartment and (2) retards the reduction of these organisms in the tank.

PART II

This part of the work concerned itself with the fate of *B. coli* and *B. aerogenes* in the fresh solids that enter the tanks continuously. As indicated in the previous discussion the majority of them pass on in the effluent of the tanks with the finely suspended solids to the filter beds where they are greatly reduced in numbers. However, considerable numbers of these bacteria are accumulating with the fresh solids in the digestion compartment of the tanks. They are always found present in these solids in considerable numbers especially when the tank is in operation and is receiving large quantities of solids. What is their fate and rôle here?

A series of laboratory experiments was started with the purpose

of answering this question. The arrangement of the experiment was as follows:

1 Fresh solids diluted 1:3
2. Ripe sludge
3. Ripe sludge plus 2 per cent fresh solids
4. Ripe sludge plus 4 per cent fresh solids

To numbers 3 and 4 respectively 2 and 4 per cent of fresh solids (by volume) were added daily. The numbers of B. coli and

TABLE 5

The numbers of B. coli and B. aerogenes in the digestion of fresh solids alone and in combination with sludge

	I FRESH SOLIDS		II SLUDGE		III SLUDGE + 2 PER CENT FRESH SOLIDS		IV SLUDGE + 4 PER CENT FRESH SOLIDS	
	Coli	Aero-genes	Coli	Aero-genes	Coli	Aero-genes	Coli	Aero-genes
	thousands	thousands	thou-sands	thou-sands	thou-sands	thou-sands	thou-sands	thou-sands
September 24	1,000	100	100	100				
September 25	1,000	500						
September 26	10,000	10,000	10	10	10	10	50	50
September 30	500	500	10	10	50	50	50	50
October 3 ..			0 1	0 1	10	50	100	100
October 7 .	100	100	0	0	10	1	50	10
October 10 . ..	0 1	0 1	0	0	50	10	50	50
October 14	5	5	0.1	0 1	5	5	100	1
October 21....	10	10	0	0	5	1	5	50
November 11. . .	0 1	0	0	0	10	1	10	10
November 18 ..					5	1	10	10

B. *aerogenes* were determined at intervals. The results are given in table 5. It can be seen at a glance that the numbers of B. *coli* and B. *aerogenes* follow each other closely. The number of these organisms in the fresh solids increased up to ten million after two days incubation and within a week they were back to their original numbers whereas after two weeks they fell to a very low level. If we compare the numbers of these organisms with total acidity production in the digestion of fresh solids as given by Rudolfs et al. (1924) we find a very close relationship. Acidity increases tremendously in the first two or three days after which

it increases rather gradually and with fluctuations up to three weeks when it begins to drop. It is natural to ascribe this initial and sudden increase in the acidity to the activities of these intestinal organisms which have a very decided carbohydrate metabolism. The soluble carbohydrates of the fresh solids offer a good medium for their activities as a result of which large quantities of acids are produced. This high acid concentration in turn checks and represses their further growth. The reduction of the acidity can result from neutralization by alkaline material produced in the course of digestion or from the direct breaking down of organic acids by other groups of organisms. It is also possible that a combination of these two factors operate in the same direction.

In the second part of this experiment, where different amounts of fresh solids were added daily to ripe sludge, the tendency was similar except that the numbers did not go to as low a level. In the sludge bottle to which no fresh solids were added the numbers of *B. coli* and *B. aerogenes* were naturally reduced to a low level within a week. With 2 per cent fresh solid additions the maximum numbers occurred within the same period of time after which the numbers fluctuated with a declining tendency until an equilibrium was established. With 4 per cent fresh solids the maximum numbers occurred after ten days after which the numbers decreased with fluctuations until an equilibrium was established which was higher than with two per cent fresh solids.

The significant part of this experiment is that even with daily additions of fresh solids the numbers of *B. coli* and *B. aerogenes* do not keep on increasing but after reaching a maximum within a week or so they begin to decrease until they reach an equilibrium. The level of this equilibrium depends upon the amount of material added.

The conclusion reached as to the effect of materials produced in the course of digestion of fresh solids on the intestinal organisms applies in this case where fresh solids are added daily to ripe sludge. In fact each daily addition of fresh solids can be taken as a miniature portrait of what is taking place when fresh solids are digested alone. Each daily addition of fresh solids carries

with it a number of the intestinal organisms which act on the available carbohydrates of the fresh material, resulting in an increase of acidity. Their numbers increase until the acidity reaches a certain point and then they are repressed in spite of the constant addition of fresh solids. The process in this case is complicated because of the action of ripe sludge in producing simultaneously alkaline material which over-balances the initial increase in acidity to a certain extent. The greater the addition of solids the greater the available food, hence the higher the peak attained will be. The numbers from this point on will be controlled by the balance between acid production of the neutralizing effect of sludge, which balance determines the point of equilibrium for these organisms.

Summary and conclusions

When fresh solids are digested without seeding the numbers of *B. coli* and *B. aerogenes* rise to a maximum within two days after which they fall rapidly to a low level. This coincides with the increase in acidity due to the attack of available carbohydrates. It is suggested that the *B. coli* group is mainly responsible for the decomposition of available carbohydrates in the beginning, giving rise to high acidity which in turn checks their own numbers.

With daily additions of fresh solids to ripe sludge the same relationship holds, namely, an increase in *B. coli* and *B. aerogenes* to a peak within a week or so, the level of the peak depending on the amount of fresh solids added. Their numbers decrease after this until an equilibrium is reached, the level of which is also controlled by the amount of material added.

REFERENCES

RUDOLFS ET AL. 1924 Studies in the biology of sewage disposal. N. J. Agr. Exper. Sta. Bul. 403.
SKINNER, C. E, AND MURRAY, T. J. 1924 Jour. Infect. Dis , **34**, 585–591.

THE DIFFUSION PRODUCTS OF BACTERIAL CELLS AS INFLUENCED BY THE PRESENCE OF VARIOUS ELECTROLYTES[1]

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INTRODUCTION

The influence of electrolytes upon plant and animal cells has been studied by a great number of observers; and the literature of the subject has been so well reviewed by Falk (1923) that it requires no detailed summary here. In general the influence of the electrolytes has been measured by the growth of cells in favorable media or their survival in unfavorable media or by the rate of chemical change produced through cell activity, in the formation of carbon dioxide from sugar solutions, in the formation of ammonia from pepton or in the process of nitrification. Changes in electrical resistance have also been studied by many observers, with contractility of muscle and viability of eggs and larvae in animal forms. These investigations have, as is well known, justified the broad conclusions that dilute solutions of electrolytes are favorable and stronger solutions unfavorable, to biological processes, that bivalent cations are more powerful in their effects than monovalent cations, that where permeability effects are manifest the univalent cations tend to increase diffusion and the bivalent cations (in similar concentration) tend to decrease it, and that these two types of cations appear to exhibit a more or less definite antagonism.

Since the appearance of Falk's review of the subject the most

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[1] Part of a dissertation presented by H. J. S. for the degree of Doctor of Philosophy in Yale University.

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important contributions have been along four different lines as follows:

a. Cook (1926) has shown that the production of carbon dioxide by Aspergillus is decreased by H, Cu and Hg ions in the concentrations studied while Ag first increases and then decreases it.

b. The penetration of various substances into the vegetable cell has been studied by direct observation in the case of the giant cells of Nitella and Valonia by Osterhout (1925), Osterhout and Dorcas (1925), Irwin (1926), and M. M. Brooks (1926 a, b). The first three observers claim that the plant cell is penetrated only by undissociated molecules, a conclusion which Mrs. Brooks denies.

c. Various investigators have reported on the effect of electrolytes upon the electrical conductivity of suspensions,—Peters (1908), studying Paramecium; Stiles and Jorgenson (1915) and S. C. Brooks (1917), the tissues of higher plants; Medes and McClendon (1920), Elodea; Gray (1920, 1921), trout eggs; Osterhout (1922), Laminaria; Shearer (1919, 1920), Green and Larson (1922), Johnson and Green (1924), MacDougall and Green (1924), Brooks (1925) and Zoond (1927), bacterial cells. There is considerable question as to the interpretation of much of this work and as Brooks, Green and Larson and others have emphasized increased conductivity may be merely due to exosmosis of electrolytes. Be this as it may it appears that monovalent cations generally cause an increase followed by an irreversible fall. Osterhout and Shearer believe that the dead cell has no resistance but Green and Larson and Zoond report only a very moderate decrease.

d. Finally, a large group of recent workers have studied the effect of electrolytes upon the passage of various anions and cations into and out of erythrocytes. Among the more important of the studies of this type are those of Gürber (1895), Hamburger (1916), De Boer (1917), Van Slyke and Cullen (1917), Fridericia (1920), Doisy and Eaton (1921), Mukai (1921), Wiechmann (1921), Mellanby and Wood (1923), Ashby (1924) and Coulter (1924). In general these observers find that H, Cl and CO_2 migrate very readily back and forth between corpuscles and

serum while Na and K according to most investigators (but not Mellanby and Wood and Ashby), fail to do so.

The present study involves phenomena related in a measure to the last two types of investigation reviewed above. It deals however with specific chemical products of diffusion and not with change in conductivity and with bacterial cells and not erythrocytes. We have sought to determine the effect of electrolytes upon the nature and amount of the products which diffuse outward from the bacterial cell into a surrounding menstruum in which the organisms are either barely surviving or slowly dying out but without active growth processes on the one hand or the action of powerfully toxic agents on the other. It was thought that a study of this type would perhaps throw light upon the question of the influence of electrolytes upon normal metabolism and the behavior of the cell membrane in normal metabolism as distinct from their effect upon enzyme action as manifest in such processes as fermentation or ammonification.

In a previous communication from this laboratory (Shaughnessy and Falk, 1924) it was shown that the cells of *Bact. coli* exert a marked buffering effect when suspended in distilled water, an effect which rises to a maximum in the range favorable to viability (pH 6.0 to 6.9) and which gradually falls off with increasing acidity or alkalinity. Neutral salts (Ca and Na) greatly decrease this buffer action particularly on the alkaline side, although in highly toxic concentration of calcium there is a secondary increase in buffer, probably due to lysis and liberation of buffer substances from the ruptured cell.

This power of the cell to regulate the hydrogen-ion concentration of a surrounding menstruum is one of its most fundamental biological properties. This power is in part exerted by the binding of positive or negative ions by the cell as a whole. It is in part, however, due to changes produced in the adjacent menstruum by the bacterial cells through the discharge of substances into that menstruum. Such a process was demonstrated by Winslow and Falk (1923) who showed that in alkaline solution the bacteria liberate acidic substances which create a zone of lower alkalinity in their immediate vicinity.

In the earlier work a buffering power conducted in this laboratory no distinction could be made between these processes since the influence of the cells upon the menstruum was determined in the presence of the cells which exerted it. The purpose of the present study was to discover the nature of the changes produced in the surrounding medium by analysis of the menstruum after the cells themselves had been removed by centrifuging.

GENERAL TECHNIQUE EMPLOYED IN OUR EXPERIMENTS

Two types of bacteria were used for the present study, one a strain of *Bact. coli* and the other a strain of *B. cereus*. Both have been extensively used in previous investigations made in this laboratory and they have been found to differ from each other in one highly important respect. The *Bact. coli* survives in practically undiminished numbers in distilled water or dilute salt solutions throughout a moderate pH range while the *B. cereus* dies out very rapidly in any such menstruum (not containing organic protective substances) ninety per cent of the cells being non-viable after an hour. We were thus able to compare the reactions of living and dead cells as exemplified by these two types.

The *Bact. coli* was cultivated before each test for twenty-two to twenty-four hours at 37°C. on nutrient agar in Kolle flasks; the *B. cereus* on the other hand was cultivated for fourteen to sixteen hours at 22° to 24°C. (a condition which previous studies had shown would ensure freedom from spores). The organisms were then washed off in distilled water, filtered through cotton to remove any trace of agar, centrifugalized twice at high speed from water and finally resuspended in water. The final suspensions were examined microscopically to make sure of cultural purity and to prove that there were no spores. The concentration of the suspension was then standardized against turbidity standards made from $BaCl_2$ and H_2SO_4 in varying proportions.

The water was freshly distilled from a Barnstead still and gave no test for ammonia with Nessler's reagent. It rapidly reached an equilibrium with the CO_2 of the atmosphere so that its acidity was consistently at pH 5.7 to 5.8. The NaCl was

Baker's Analyzed and the $CaCl_2$ was the same brand, recrystallized from water to rid it of $MgSO_4$. These chemicals were then dried in an electric oven at 105°C. and kept in a desiccator until weighed.

The HCl solutions used for titration and for adjusting the pH of the solutions was standardized by the potentiometric method, using the apparatus described by Shaughnessy and Falk (1924). The NaOH solution used for the titrations was standardized against the HCl solution daily.

All glassware used in these experiments was of Pyrex brand, neutralized and cleaned each time by immersion in sulphuric acid-potassium dichromate solution over night and by subsequent rinsing with running hot tap water with final rinsing in distilled water. It was then thoroughly dried in an electric oven at 105°C. for three to four hours.

EFFECT OF DIFFUSION PRODUCTS UPON THE REACTION OF THE MENSTRUUM

In the conduct of a typical experiment, 1 cc. of a heavy suspension of the organism was added to each of a series of 150 cc. Erlenmeyer flasks, containing exactly 50 cc. of the test solution, which had already been adjusted in bulk to the desired pH with HCl or NaOH,—giving a final concentration of about 500 million cells per cubic centimeter. For each flask of suspension there was also a control flask containing 50 cc. of the test solution of the same pH plus 1 cc. of distilled water. The flasks were very lightly plugged with cotton to prevent the type of autolysis that Jaumain (1922) has observed to occur in tightly plugged tubes and were kept in the incubator at 37°C. for the specified lengths of time. The control flasks were kept under the same conditions as the respective suspensions but were not centrifugalized. The zero time period in the charts and tables corresponds to analyses made from flasks centrifugalized immediately after seeding.

After the organisms were strongly sedimented in the centrifuge, the supernatant fluid was carefully poured off, without disturbing the sediment, into Erlenmeyer flasks of the same size as those containing the control. A measured amount of one of

Clark and Lubs' series of indicators was added to the test and control fluids, respectively, the pH of each recorded and the pH of the test fluid adjusted to that of the control with N/1000 HCl or NaOH as the case might be.   In our charts and tables we have given the figures in terms of titratable acid or alkali necessary to cause this change because it was not always possible to measure the pH of the test fluid with the same indicator that was used for the control and because pH values cannot be averaged easily because of their logarithmic nature.   In some cases we have made note that the total acid or alkali neutralized and the pH changes fail to harmonize and have indicated our explanation of the phenomenon.

The solutions studied included water and CaCl₂ and NaCl solutions (in 0.0145, 0.145 and 1.45 M concentration) adjusted to pH values of 6.0, 7.0 and 8.0.   The periods of exposure studied were 0, $\frac{1}{2}$, 1, 2, 4 and 24 hours.   Each figure presented in the tables is the average of from two to seven different experiments, the exact number being given in the table in each case.

In addition to the studies made with living *Bact. coli* and with *B. cereus* cells which had died from exposure to an aqueous menstruum we also made some observations on the boiled cells of both species and on *Bact. coli* cells killed by 15 minute exposure to a temperature of 60°C. which we found by plating to be the minimum time in which the organism could be killed at that temperature.   The volume of the suspension was of course restored to its original value after heating.

The results of our tests upon *Bact. coli* and *B. cereus* in their normal state and after heating are presented in tables 1 to 8. The data are grouped in the tables according to the organism tested and the reaction of its original menstruum but it will perhaps be more instructive to discuss the results in a different order, taking up first all tests made with water as a menstruum and then considering the effect of electrolytes upon the fundamental reactions involved.

In figure 1 we have presented all the important data which illustrate the effects of the various organisms tested upon the titratable acidic and alkaline substances in water without the

presence of electrolytes. The three sets of curves show reactions
in water primarily adjusted to pH values of 8.0, 7.0 and 6.0,
respectively. The ordinates above the base lines represent the
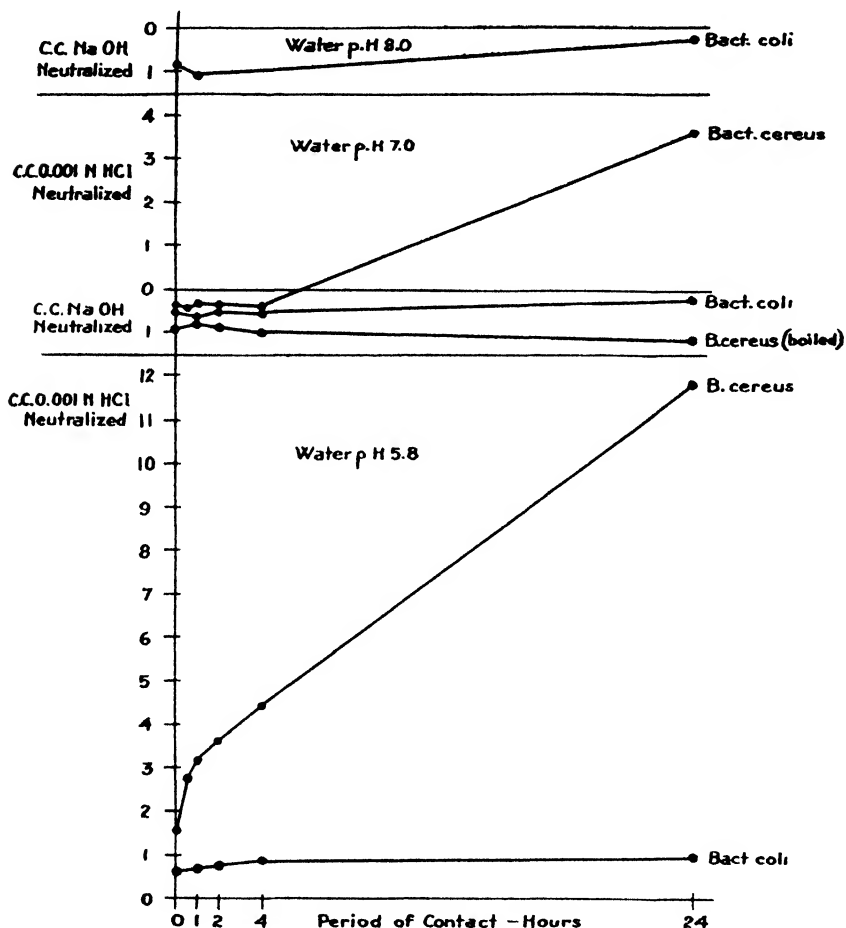
FIG. 1. THE EFFECT OF B. CEREUS AND BACT. COLI UPON THE
TITRATABLE ACIDITY OF DISTILLED WATER

amounts of acid necessary to restore the solutions to their original
values (or to the value of a control not exposed to bacterial cells).
Ordinates below the base line represent amounts of alkali necessary
to regain the original pH value.

It will be noted from figure 1 that in alkaline and neutral solution the menstruum in which the bacterial cells have been suspended becomes during the first four hours more acid so that

TABLE 1

Diffusion products of B. cereus in acid menstrua

Alkaline substances formed. In terms of cubic centimeters 0.001 N HCl necessary to neutralize 50 cc.

SOLUTION	PERIOD OF CONTACT						NUMBER OF EXPERIMENTS
	0 hour	½ hour	1 hour	2 hours	4 hours	24 hours	
Distilled water..	1.55	2 71	3.21	3.65	4 43	11.78	5
0.0145 M NaCl.	0 87	1 77	2.50	3.08	5.18	18.09	5
0.145 M NaCl	0 70	0.90	1.22	1.60	2.12	8 62	5
1.450 M NaCl....	0 76	1.19	1.25	1 34	1 31	1.36	5
0.0145 M CaCl₂	0 21	0.23	0 42	0 65	1.45	3.77	6
0.145 M CaCl₂......... ..	0.13	0 01*	0.05*	0.08*	0.10*	0.04	4
1 450 M CaCl₂.......	0.51	0.39	0.34	0 00	0.00	0 13*	3

All solutions started at pH 5.7 to 5.8

* Acidic substance—neutralized with 0.001 N NaOH.

TABLE 2

Diffusion products of B. cereus in neutral menstrua

Acidic substances formed. In terms of cubic centimeters 0.001 N NaOH necessary to neutralize 50 cc.

SOLUTION	PERIOD OF CONTACT						NUMBER OF EXPERIMENTS
	0 hour	½ hour	1 hour	2 hours	2 hours	24 hours	
Distilled water..	0.38	0.43	0 37	0.38	0.44	3.55*	6
0 0145 M NaCl.	0 95	1.20	1.38	1.76	1.73	4.97*	4
0.145 M NaCl..	1.29	2.24	2.08	2.24	2.33	1.14*	6
1.450 M NaCl.....	0.82	0.80	0.65	0.48	0.66	0.55	5
0.0145 M CaCl₂	0.84	1.02	0.96	0.95	0.71	0.28*	3
0.145 M CaCl₂.....	0.72	0.89	1.00	0.96	0.91	0.32	7
1.450 M CaCl₂...............	1.09	1.65	1.52	1.40	2.03	1.86	3

All solutions started at pH 6.8 to 7.0.

* Alkaline substances—neutralized with 0.001 N HCl.

it requires the addition of alkali to bring the solution back to the starting point. In the case of an initially alkaline solution exposed to *Bact. coli* the effect is particularly striking. A wholly

different effect is however manifest after periods longer than 4 hours. At the twenty-four-hour period production of acid is overbalanced by a production of alkali, moderate in the case of

TABLE 3

Diffusion products of Bact. coli in acid menstrua

Alkaline substances formed. In terms of cubic centimeters 0 001 N HCl necessary to neutralize 50 cc.

SOLUTION	PERIOD OF CONTACT					NUMBER OF EXPERI- MENTS
	0 hour	1 hour	2 hours	4 hours	24 hours	
Distilled water	0 63	0 70	0 72	0 85	0 93	5
0.0145 M NaCl	0 49	0 50	0 83	0 78	1.69	4
0 145 M NaCl	0 29	0 28	0 48	0 65	1 86	4
1.450 M NaCl	0 09	0 02*	0 00	0 00	0 11*	5
0 0145 M CaCl₂	0 29	0 34	0 32	0 33	0 74	5
0.145 M CaCl₂	0 40	0 48	0 46	0 45	0 82	3
1.450 M CaCl₂	1 15	1 14	1 06	0 54	0 12	3

All solutions started at pH 5 7 to 5.8.
* Acidic substances—neutralized with 0 001 N NaOH.

TABLE 4

Diffusion products of Bact. coli in neutral menstrua

Acidic substances formed. In terms of cubic centimeters 0. 001 N NaOH necessary to neutralize 50 cc.

SOLUTION	PERIOD OF CONTACT					NUMBER OF EXPERI- MENTS
	0 hour	1 hour	2 hours	4 hours	24 hours	
Distilled water	0 49	0 63	0 46	0 45	0 24	4
0.0145 M NaCl	0 30	0 36	0 49	0 57	0 76	5
0.145 M NaCl	0.53	0 64	0 63	0 55	0 24	3
1.450 M NaCl	0 57	0 58	0 56	0 58	0 52	3
0.0145 M CaCl₂	0 47	0 55	0 49	0.56	0 46	3
0.145 M CaCl₂	0 55	0 55	0 46	0 42	0.11	3
1.450 M CaCl₂	1 12	1.61	1 39	1 45	0 43	4

All solutions started at pH 6.8 to 7.0.

Bact. coli and much more marked in the case of *B. cereus*, the reaction in the latter case actually reaching a pH of 7.4.

It may be noted that these studies in the alkaline medium were rendered very difficult by the continued absorption of

carbon dioxide from the atmosphere.   Since our results represent differences between titratable acidity in menstrua containing bacterial diffusion products and in control solutions of the same initial reaction the results are, however, believed to be approximately accurate.

TABLE 5

Diffusion products of Bact. coli in alkaline menstrua

Acidic substances formed.   In terms of cubic centimeters 0.001 N NaOH necessary to neutralize 50 cc.

SOLUTION	PERIOD OF CONTACT			NUMBER OF EXPERIMENTS
	0 hour	1 hour	24 hours	
Distilled water	0.89	1 09	0 31	5
0 0145 M NaCl	1 27	0.77	0 15	2
0 145 M NaCl	1 24	1.32	0 47	2
1 450 M NaCl	1 35	1.30	0.33	2
0 0145 M CaCl$_2$	1 36	1 18	0.42	3
0 145 M CaCl$_2$	0.99	0.90	0 25	2
1 450 M CaCl$_2$	3.33	2 86	2 15	2

All solutions started at pH 8.0 to 8.1.

TABLE 6

Diffusion products of heat-killed cells of Bact. coli in neutral menstrua

Acidic substances formed.   In terms of cubic centimeters 0 001 N NaOH necessary to neutralize 50 cc.

SOLUTION	PERIOD OF CONTACT				NUMBER OF EXPERIMENTS
	0 hour	1 hour	4 hours	24 hours	
Distilled water	0 52	0 46	0.42	0 26	3
0.0145 M NaCl	0.46	0.42	0.40	0 47	3
1.450 M NaCl	0 52	0 59	0.47	0 58	3
1 450 M CaCl$_2$	1 26	1.43	1.57	0 61	3

All solutions started at pH 6.8 to 7.0.

In the acid solutions the cells of both species cause an immediate and marked production of alkaline substances and this production of alkaline substances continues during the whole period of the experiment, slowly in the case of *Bact. coli* and very rapidly in the case of *B. cereus*.

Heat-killed and even boiled cells of *Bact. coli* (omitted from

the chart; see tables 6 and 7) exert just the same effect as do the living cells but the effect of boiling upon *B. cereus* is to increase the initial production of acidic substances and to check altogether the later reversion to a strongly alkaline reaction.

While preferential absorption of ions may of course play some part in these phenomena, it is clear that we must be dealing

TABLE 7

Diffusion products of cells of Bact. coli, boiled for thirty minutes, in neutral menstrua

Acidic substances formed   In terms of cubic centimeters 0.001 N NaOH necessary to neutralize 50 cc.

SOLUTION	PERIOD OF CONTACT				NUMBE OF EXPERI MENTS
	0 hour	1 hour	4 hours	24 hours	
Distilled water	0 59	0 43	0 72	0 20	3
0 0145 M NaCl	0 57	0 38	0 61	0 52	3
1 450 M NaCl	0 82	0 72	1 00	0 40	3
1 450 M CaCl$_2$	3 54	2 00	3 51	3.33	3

All solutions started at pH 6 8 to 7.0.

TABLE 8

Diffusion products of cells of B. cereus, boiled for thirty minutes, in neutral menstrua

Acidic substances formed.   In terms of cubic centimeters 0.001 N NaOH to neutralize 50 cc

SOLUTION	PERIOD OF CONTACT				NUMBER OF EXPERI- MENTS
	0 hour	1 hour	4 hours	24 hours	
Distilled water	0 96	0 76	0 96	1 07	2
0 0145 M NaCl	1 03	1 37	1 54	0 74	2
1 450 M NaCl	1 80	1 32	1.58	1.49	2
1 450 M CaCl$_2$.	3 82	3 83	2 75	3 32	2

All solutions started at pH 6.8 to 7.0

chiefly with the actual elimination into the menstruum by the cell of acidic and basic substances respectively.[2]   It appears that the first tendency is toward a liberation of acid substances in a

[2] It has been shown in earlier work from this laboratory (Shaughnessy and Falk, 1924) that the alternative explanation (fixation of basic or acid substances, cannot be in all cases invoked.

neutral or alkaline solution and of basic substances in an acid solution, a process tending to regulate the reaction of the menstruum to the point which we know to be optimum for the life of bacterial cells,—the process described by Winslow and Falk (1923). There seems a close parallelism here between our results and those obtained by Coulter (1924) with red blood cells (an increase in acidity followed by an increase in alkalinity), although his explanation of the phenomenon is different from ours. The second process (alkali production), unlike the first, is checked by boiling.

NATURE OF THE DIFFUSION PRODUCTS WHICH CONTROL THE REACTION OF THE MENSTRUUM

At this point it is worth while to consider the nature of the diffusion products which lead to the changes in reaction which have been discussed. We should naturally expect that carbon dioxide and diffusion of hydrogen alone or in combination with inorganic anions, on the one hand and ammonia on the other hand, would play a part in the changes toward an acid and an alkaline reaction respectively.

a. Hydrogen. The first possible source of acidity would appear to be hydrogen, diffusing out from the cell in combination with basic ions. As an indirect measure of this type of reaction we estimated the chlorides and phosphates contributed to the menstruum by the bacterial cells.

Chlorides were measured by the micro method of Van Slyke (1923) modified so that we used 50 cc. of test fluid and made our determinations with 0.005 N $AgNO_3$ and 0.005 N KCNS in place of the stronger concentrations. The sulfocyanate was checked daily against the silver nitrate and a correction factor made.

The results are presented in tables 9 and 10. It will be noted that in distilled water the diffusion of chlorides is highly variable and never great in amount; but that it is perhaps most marked in an acid menstruum.

Phosphates were measured by the method, credited to Benedict, described in Meyers' (1924) monograph on blood analysis. It consists in clarification of the test fluid with trichloracetic acid,

heating with hydroquinone-bisulfite and molybdic acid reagents and comparison of the cooled solution with a standard phosphate. We modified the procedure so that we could use 25 cc. of the supernatant fluid and control (and still get a good color for comparison) by substituting a phosphate standard made so that 1 cc. = 0.01 mgm. of phosphate. We used 0.0, 0.1, 0.3, 0.5, 0.7, etc., up to 2.5 cc. of this standard diluted to 25 cc. as stand-

TABLE 9

Diffusion products of B. cereus

Chlorides. Expressed as cc. 0.005 N AgNO₃ to react with 50 cc.

SOLUTION	PERIOD OF CONTACT				NUMBER OF EXPERIMENTS
	0 hour	1 hour	4 hours	24 hours	
Distilled water (pH 5 7)	0 066	0 049	0 075	0 013	3
Distilled water (pH 7.0). .. .	0 110	0 129	0 139	0.018	3
0.0145 M NaCl (pH 7.0) . .	0 351	0 333	0 179	0 342	3

TABLE 10

Diffusion products of Bact. coli

Chlorides. Expressed as cubic centimeters 0.005 N AgNO₃ to react with 50 cc.

SOLUTION	PERIOD OF CONTACT				NUMBER OF EXPERIMENTS
	0 hour	1 hour	4 hours	24 hours	
Distilled water (pH 5.7) .	0 013	0 064	0 052	0 074	3
Distilled water (pH 7.0)..	0 007	0 012	0 005	0.002	3
0.0145 M NaCl (pH 7.0)	0.182	0.320	0 263	0 322	3

Cells killed by heating at 60°C. for fifteen minutes

Distilled water (pH 5.7)..	0 039	0 069	0 079	0 074

ards in Nessler tubes. Fresh standards were made up daily and, as each group of test fluids at any time period was heated, one new standard was also made to check the equivalent standard in the set already made. By this method we were able to determine 0.001 mgm. of phosphate readily.

The results for distilled water (see tables 11, 12 and 13) show as in the case of chlorides an almost insignificant increase in basic ions. It would seem from these data that in the absence

of added electrolytes the changes in the menstruum effected by the bacterial cell in the direction of acidity are not in large

TABLE 11

Diffusion products of B. cereus

Phosphates. Milligrams per 25 cc.

SOLUTION	PERIOD OF CONTACT				NUMBER OF EXPERIMENTS
	0 hour	1 hour	4 hours	24 hours	
Distilled water (pH 5 7)	0 001	0 002	0 004	0 007	3
Distilled water (pH 7 0)	0 001	0.003	0 006	0.010	3
0 0145 M NaCl (pH 7 0)	0 002	0 003	0 016	0 019	3
1 450 M NaCl (pH 7 0)	0 001	0 004	0 009	0 011	3

TABLE 12

Diffusion products of Bact coli

Phosphates. Milligrams per 25 cc.

SOLUTION	PERIOD OF CONTACT				NUMBER OF EXPERIMENTS
	0 hour	1 hour	4 hours	24 hours	
Distilled water (pH 5 7)	0 001	0 001	0 001	0 003	3
Distilled water (pH 7 0)	0 001	0 002	0 002	0 002	6
0 0145 M NaCl (pH 7 0)	0 003	0 002	0 003	0 008	4
1 450 M NaCl (pH 7 0)	0 001	0 001	0 002	0 005	3
0 0145 M CaCl$_2$ (pH 7 0)	0 000	0 002	0 002	0 001	3

TABLE 13

Diffusion products of cells of Bact. coli, boiled for thirty minutes, in neutral menstrua

Phosphates. Milligrams per 25 cc.

SOLUTION	PERIOD OF CONTACT				NUMBER OF EXPERIMENTS
	0 hour	1 hour	4 hours	24 hours	
Distilled water	0.001	0 002	0 003	0 003	2
0.0145 M NaCl	0 002	0 003	0 004	0 006	3
1 450 M NaCl	0.001	0.002	0 002	0 000	3

All solutions started at pH 6 8 to 7.0.

measure due to diffusion of hydrogen in combination with basic ions. Chlorides and phosphates were selected for this study because they are the only anions present in appreciable amount

which would be likely to carry hydrogen out from the cell. Guillemin and Larson (1922) have shown that the SO_4 ion is present in bacterial cells in negligible amount.

It appears from these results that the processes of diffusion and the regulative action upon the menstruum must be quite different in bacteria and in red blood cells,—as one might naturally expect would be the case. Gürber (1895), Hamburger (1916), DeBoer (1917), Van Slyke and Cullen (1917), Fridericia (1920), Doisy and Eaton (1921), Wiechmann (1921), Mukai (1921) and Mellanby and Wood (1923) and Coulter (1924) all maintain that chlorin passes in some form rather readily into and out of the erythrocyte. Osterhout (1922) and Irwin (1923a) on the other hand find that Nitella cells are not easily penetrated by chlorides which certainly seems to be the case with the bacteria.

b. Carbon dioxide. The study of carbon dioxide as a factor in regulating the reaction of a solution is surrounded by very great difficulties on account of the constant adjustment which takes place between the concentration of this substance in solution and in the adjacent atmosphere. Furthermore, the course of this process is also materially influenced by the presence of electrolytes in the solution itself, a fact which probably accounts for some of the phenomena observed by Winslow and Falk. The three solutions which best illustrate the effect of buffer upon viability are water, 0.145 M NaCl and 0.145 M CaCl₂. We find that if these three solutions be adjusted to pH 8.0 and allowed to stand, without bacterial cells present, for one hour their reactions will change respectively to 7.3, 7.3 and 7.8 under the direct influence of atmospheric CO_2 alone.

For these reasons we decided that it would be useless to attempt any direct quantitative measurements of carbon dioxide and resorted instead to the indirect process of removing the carbon dioxide present by blowing carbon-dioxide free air through the medium and then determining the pH at equilibrium in the carbon-dioxide-free solution, an equilibrium presumably determined by mineral acids. This process would of course remove other acids than carbon dioxide if they were of a volatile or readily oxidizable nature. The extensive oxidation of any acids

present in a period as short as one hour is exceedingly improbable. The introduction of volatile bases was prevented by drawing the incoming air through concentrated sulphuric acid.   Our complete equipment consisted of a train of four wash bottles containing respectively soda lime, concentrated NaOH, concentrated H_2SO_4 and distilled water, through which we bubbled compressed air before it was admitted to the test solution.

Another factor came to the surface as soon as we attempted to measure the change in acidity of the solutions.   We found that our test and control solutions not only returned to the control pH (neutrality) but became alkaline.   The reason for this was not long in disclosing itself.   Our distilled water before adjustment was at pH 5.8 due to equilibrium with the atmospheric CO_2 (a fact noted by Loeb, 1922, as well as by many others) and, when we brought it to neutrality, we had created a solution containing $NaHCO_3$ and perhaps Na_2CO_3.   At any rate, when we blew off CO_2, the NaOH was again liberated and made the solution alkaline.   It was possible, however, to compare the solutions in regard to the final pH reached.

By this method we found that water, 0.0145 M NaCl and 1.450 M NaCl which had been in contact with either *Bact. coli* or *B. cereus* came to the same pH equilibrium point (measured colorimetrically) that the controls did.   1.450 M $CaCl_2$ alone became less alkaline by 0.1 to 0.2 pH units than its control.   We shall return to this last phenomenon later.

The net result of this part of our study was to indicate that the acidity produced in a surrounding menstruum by the bacterial cell is only in small measure due to the diffusion of hydrogen ions, alone or in combination with mineral anions, but is chiefly due to volatile acidic compounds of which carbon dioxide is undoubtedly the chief representative.

c. Ammonia.   In considering the source of alkaline changes in the menstruum studied the first logical point of attack would seem to be ammonia.   Coulter (1924) believed that such a change in his experiments was caused by ammonia production while Brooks (1923b) believed it to be due to diffusion of basic ions; neither had succeeded in demonstrating either fact.

It was apparent that it was useless as well as impossible in the time available to measure the production of ammonia in all the solutions studied.   For that reason we selected for this and for all

TABLE 14

Diffusion products of B. cereus

Ammonia nitrogen.   Milligrams per 50 cc.

SOLUTION	PERIOD OF CONTACT				NUMBER OF EXPERIMENTS
	0 hour	1 hour	4 hours	24 hours	
Distilled water (pH 5 7)	0 006	0 009	0 011	0.070	3
Distilled water (pII 7 0).	0.008	0 009	0 018	0 090	3
0 0145 M NaCl (pH 7.0)	0 009	0 014	0 035	0 150	3
1.450 M NaCl (pH 7.0)	0.000	0 000	0 000	0 000	3

TABLE 15

Diffusion products of Bact. coli

Ammonia nitrogen.   Milligrams per 50 cc.

SOLUTION	PERIOD OF CONTACT				NUMBER OF EXPERIMENTS
	0 hour	1 hour	4 hours	24 hours	
Distilled water (pH 5 7) . .	0 004	0 006	0 009	0.019	3
Distilled water (pII 7 0)	0 005	0 006	0 008	0 020	3
0 0145 M NaCl (pII 7 0)	0 007	0 008	0.011	0 034	3
1.450 M NaCl (pII 7 0)	0 000	0 000	0 000	0 000	3

TABLE 16

Diffusion products of cells of Bact. coli, boiled for thirty minutes, in neutral menstrua

Ammonia nitrogen.   Milligrams per 50 cc.

SOLUTION	PERIOD OF CONTACT				NUMBER OF EXPERIMENTS
	0 hour	1 hour	4 hours	24 hours	
Distilled water.....	0 002	0.002	0.002	0.002	3
0.0145 M NaCl....................	0.003	0.003	0.004	0.012	3
1.450 M NaCl..	0.000	0.000	0 000	0.000	2

All solutions started at pH 6.8 to 7.0.

of our later work the two salts which showed the greatest changes, i.e., 0.0145 M NaCl and 1.450 M NaCl, at neutrality and water at acid and neutral reaction.   It was found that the CaCl₂ solutions

precipitated with the Nessler reagent so it was necessary to use 1.450 M NaCl instead of the same strength of $CaCl_2$.

The measurement of ammonia production was made by the method of direct Nesslerization given in Standard Methods of Water Analysis of the American Public Health Association, 1923, page 16, except that preliminary clarification was found to be unnecessary. The results are expressed in terms of milligrams × 10^{-3} of ammonia nitrogen for each 50 cc. of solution.

The results of these determinations are presented in tables 14 to 16. They indicate a very considerable production of ammonia when the living cells of either *Bact. coli* or *B. cereus* had been present. The smallest quantity recorded (0.004 mgm.) is equal to approximately N/200,000 solution of NH_4OH. The boiled cells of *Bact. coli* show a very marked inhibition of this ammonia formation.

It is also highly significant to note that the output of ammonia is about the same in acid and in neutral solution. The very different effect upon net reaction in these solutions must, then, be due to a balance between the production of ammonia and of carbon dioxide.

So far as *B. cereus* is concerned, it is clear that the production of ammonia must be due to the action of autolytic enzymes rather than to normal metabolism since the cells are killed by the conditions of the experiments. The important part played by NH_3 and CO_2 in the phenomena studied is in harmony with the findings of almost all those who have observed similar phenomena in either plant or animal cells. Rous (1925) has shown that, as one would expect, the tissue cells of mammals show the greatest readiness in the giving up and taking in of CO_2. Red blood cells of course exhibit the same phenomena in high degree. Brooks (1923a), working with Valonia cells demonstrated the ready passage of NH_3 and CO_2. Harvey (1911, 1913, 1914) showed that ammonia readily diffused into the cells of Elodea, Spirogyra and Paramoecium and McCutcheon and Lucke (1924) and Irwin (1925) have confirmed the penetration of NH_3 into starfish eggs, Gonionemus and Nitella cells.

EFFECT OF HEAT KILLING UPON DIFFUSION FROM THE BACTERIAL CELL

While the cells of *B. cercus* (which die under the conditions of these experiments) are much more permeable than those of *Bact. coli* which survive, the diffusion of acidic substances from the bacterial cell appears to go on at about the same rate from the heat killed and boiled cells as from the living cells of *Bact. coli* (compare tables 6 and 7 with table 4 and table 8 with table 2). This seems at first somewhat surprising but Osterhout (1922) records results which demonstrate that production of CO_2 may continue or even increase after death. This process might be due to decarboxylization but seems to be very rapid for such a mechanism. The diffusion of chlorides is also uninfluenced by heating (see table 10).

The production of ammonia is on the contrary almost abolished by heating as shown by a comparison of table 16 with table 15 and the same phenomenon is apparent in the abolition of the reversion after twenty-four hours to an alkaline condition, for *B. cercus* in the last column of table 8 as compared with the last column of table 2. Evidently the liberation of ammonia is intimately related to a process which is absent in the heat killed cell but not in the cell of *B. cercus* which has died from exposure to an aqueous menstruum. Apparently the enzymes which cause liberation of ammonia operate after the natural death of *B. cercus* cells in aqueous menstrua but are destroyed by heat.

It will be recalled that Shearer (1919, 1920) found the electrical resistance of meningococci killed by exposure to unfavorable menstrua was abolished by death while Green and Larson (1922) and Zoond (1927) found only a slight decrease in heat killed cells of other bacterial species. Winslow and Willcomb (1905) found that, while heat-killed bacterial cells remain stainable, cells which die normally in an unfavorable menstruum lose their staining properties almost at once. It is clear that "death" may be accompanied by widely different types of physical change in the cell membrane under different conditions. It seems probable that when bacteria die in a slightly unfavorable menstruum

their permeability is likely to increase while heat killing produces no such effect.   On the other hand, ammonia production shows a precisely opposite effect, proceeding normally in the cells of *B. cereus* which have died normally in an unfavorable menstruum but ceasing in heat-killed cells.

THE INFLUENCE OF ELECTROLYTES UPON THE DIFFUSION PRODUCTS OF THE BACTERIAL CELL

The data so far discussed indicate that the bacterial cell in aqueous suspension first regulates the reaction of the surrounding menstruum toward an optimum point by the diffusion into the menstruum of ammonia and of carbon dioxide and other volatile acids, balanced according to the original reaction of the solution. Thereafter, there occurs a liberation of an excess of ammonia, causing a progressive swing toward alkalinity which is very marked in the case of *B. cereus* (an organism which dies out rapidly during the course of the experiment).

Our next problem concerned the influence of other electrolytes (Na and Ca) upon the course of these fundamental reactions.

Taking first the primary production of acidic substances in initially neutral or alkaline solutions we note that in the case of *Bact. coli* (tables 4, 5, 6 and 7) no important influence is exerted by any of the salts studied except the strong $CaCl_2$ (1.450 M). The latter solution causes a sharp increase in titratable acidity which, however, becomes less marked with the passage of time. We are inclined to attribute this increase in titratable acidity to the liberation of protein buffers by lysis of the cells, particularly as the pH does not show any corresponding increase.   This conclusion has been confirmed by tests for protein by biuret and Millon tests which show protein to be present in the strong calcium solutions but not in any of the others.   The later decrease in titratable acidity in these calcium solutions may perhaps be attributed to the accumulation of non-reactive surface films on the protein micellae due to contact with the solution or to later absorption of the oppositely charged ions.

Turning now to the production of alkaline substances we note that both *B. cereus* (table 1) and *Bact. coli* (table 3) in acid solu-

tions show the same general phenomena. With *B. cereus* (table 1 and fig. 2) the dilute NaCl (0.0145 м) increases the amount of alkaline substances, diffused while all the other salts decrease it
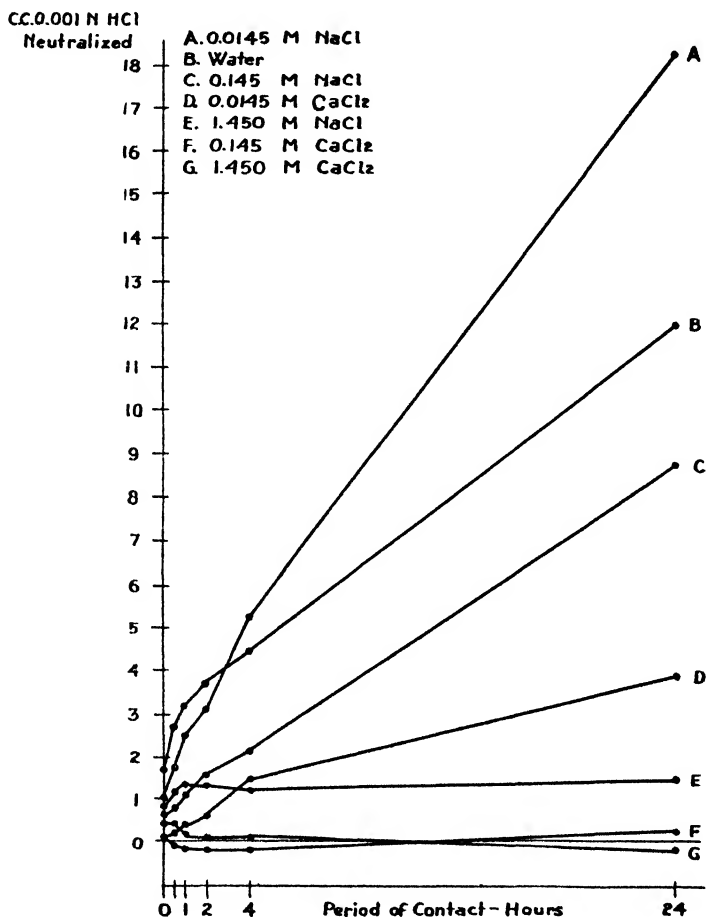
FIG. 2. THE EFFECT OF B. CEREUS UPON THE TITRATABLE ACIDITY OF ACID SOLUTIONS

in approximate proportion to their concentration, the order of effectiveness being 0.145 м Na, 0.0145 м Ca, 1.45 м Na, 0.145 м Ca and 1.45 Ca.

With *Bact. coli* (table 3 and fig. 3) the same general phenomena

appear, although both 0.145 m Na and 0.0145 Na increase diffused alkali and 0.145 Ca shows a slightly less decrease than does 0.0145 Ca. With both organisms the strong Ca solution differs from all others in showing an initial excess of alkalinity followed by a decrease, probably due to the phenomenon described above,—lysis of cells with liberation of buffer, followed by formation of surface films. The effect is more marked with *Bact. coli* than with *B. cereus* possibly because the cell wall of the latter organism is so much more permeable that it is less readily ruptured.
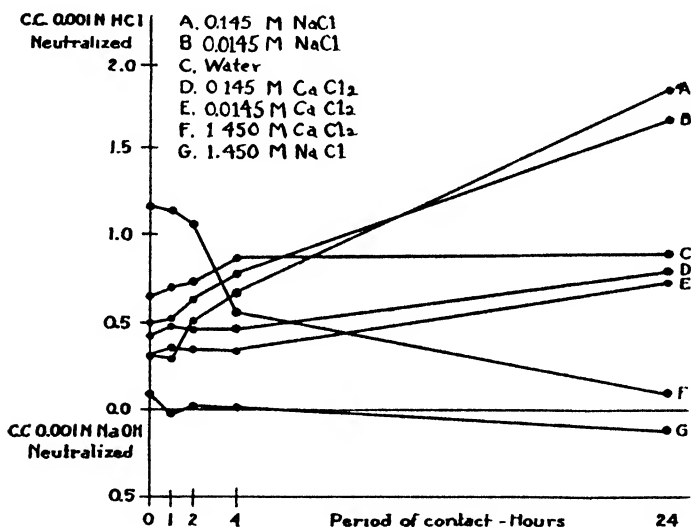
FIG. 3. THE EFFECT OF BACT. COLI UPON THE TITRATABLE ACIDITY OF ACID SOLUTIONS

The cells of *B. cereus* in neutral solution (table 2 and figure 4) show a somewhat more complex relationship but one that is easily explained on relatively simple assumptions. The aqueous solution and the solution of 0.0145 Ca, 0.145 Ca and 1.450 m Na show a progressive production of alkaline substances; most marked in the water and decreasing with the stronger salts. The 1.450 m Ca solution shows the usual initial increase in titratable acidity due to protein buffers. The weak Na solutions on the other hand exhibit a reverse curve corresponding to an

initial increase in liberation of acidic substances during the first four hours followed by a greatly increased liberation of alkali during the subsequent period. We interpret this as due to the fact that dilute sodium salts increase the permeability of the cell wall,—both to the acidic substances first set free and to
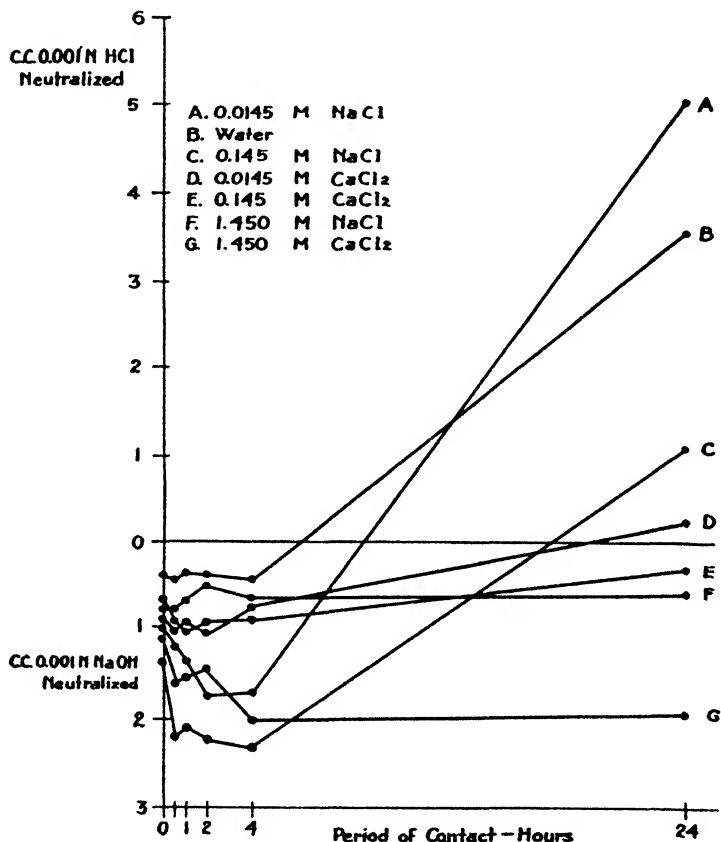
FIG. 4. THE EFFECT OF B. CEREUS UPON THE TITRATABLE ACIDITY OF NEUTRAL SOLUTIONS

the ammonia which is diffused during the later stages of the process.

The influence of electrolytes upon the production and diffusion of alkaline substances is further illustrated by the data for

ammonia, presented in tables 14, 15 and 16 and in figure 5. The weak Na solution (0.0145 M) increases the diffusion of ammonia and the strong solution (1.450 M) practically abolishes it. The latter result may be due to diminished permeability of the cell
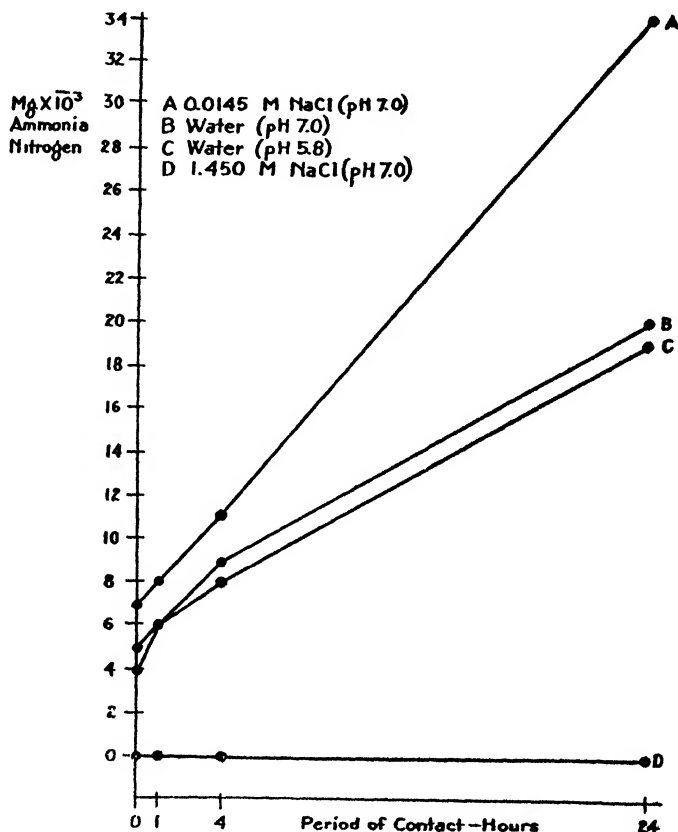
FIG. 5. THE EFFECT OF BACT. COLI UPON THE AMMONIA CONTENT OF MENSTRUA

wall or to diminished production of ammonia within the cell due to inhibition of enzyme action. Lipman (1909) has reported that production of ammonia from peptone is markedly decreased by strong NaCl.

So far as chlorides are concerned tables 9 and 10 again illustrate

the fact that a weak Na solution (0.0145 M) greatly increases the permeability of the cell wall of both *Bact. coli* and *B. cereus* as evidenced by the marked increase of chlorides present in the menstruum. It was not possible to test the effect of high concentrations of salts in the menstruum because the large initial addition of chlorides which such an experiment involves would mask any changes due to the bacterial cells.

It is of special interest to note that cells killed at 60°C. for fifteen minutes (table 10) did not show any greater diffusion of chlorides than did the normal cells, a fact opposed to the usual view that rapid exosmosis occurs upon cell death. The data for phosphates presented in tables 11, 12 and 13 show again that dilute NaCl (0.0145 M) increases diffusion.

The heat-killing or boiling of the cells produces no effect in the salt solutions different from that exerted in water,—with one single exception. In general, ammonia production is checked and carbon dioxide production unaffected in salt solutions exposed to heat killed cells as compared with those exposed to normal cells. The strong $CaCl_2$ (1.450 M) however in the case of boiled cells (table 7),—but not on the case of cells killed at 60° (table 6), shows a marked excess of acid production in comparison with living cells (table 4). We are inclined to attribute this to hydrolysis of the proteins liberated by lysis under the action of the strong calcium solution.

The general results obtained in regard to the influence of electrolytes upon the processes studied are in full accord with the findings of earlier observers. Thus, the fact that dilute solutions of electrolytes increase, while stronger solutions hinder, diffusion was demonstrated by Endler (1912) for green algae, while S. C. Brooks (1916, 1917) with dandelion tissue, Shearer (1919, 1920) with bacteria, Osterhout (1922) with Laminaria and Winslow and Falk (1923) with bacteria all report that Na tends to increase and Ca to decrease permeability. Shearer and Osterhout record a subsequent increase in permeability due to prolonged effect of the bivalent ion which may, as we suggest, be explained by actual rupture of the cell wall.

SUMMARY AND CONCLUSIONS

1. The earlier work of Shaughnessy and Falk (1924) has shown that cells of *Bact. coli* in the zone of physiological interest possess appreciable capacity to resist changes in reaction in such a manner as to avoid injury to the cell. We now find that, in addition to whatever direct absorption of H or OH ions may take place, the cells exert a distinct influence upon the reaction of the menstruum which may be measured by direct chemical tests in this menstruum after the removal of the cells. This type of action involves the liberation of acidic substances in alkaline or neutral medium and of alkaline substances in a more acid medium, the process being so balanced that the ultimate acidity approximates the zone of hydrogen ion concentration (pH 6.2 to 6.4) most favorable to viability (early results for distilled water in tables 1, 2, 3 and 4). This may be interpreted as an adaptive reaction, favorable to the life of the cell.

2. On more prolonged exposure to a somewhat unfavorable aqueous menstruum the production of ammonia overbalances that of acidic substances and in the case of the cells of *B. cereus*, which die under the conditions of the experiment, the excess of alkali liberated may be very large. This reaction is undoubtedly characteristic of injury and interference with the normal life of the cell.

3. The cells of both *Bact. coli* which survive and of *B. cereus* which die out in this menstruum appear to be relatively impermeable to chlorine and phosphate ions and also to calcium ions. The results of two series of studies on calcium have not been cited above in detail but showed insignificant amounts of this ion in menstrua exposed either to *Bact. coli* or *B. cereus*. On the other hand the cell wall allows free passage of carbon dioxide and ammonia and it is to these substances that the effects upon the menstruum are chiefly to be attributed,—the primary adaptive regulation being due to a balanced production of CO_2 and NH_3 the later alkalinity to an excess of NH_3.

4. In general the cell wall of *B. cereus* is obviously much more permeable than that of *Bact. coli*, a phenomenon probably related

to the fact that this organism promptly succumbs in aqueous suspension while *Bact. coli* survives in almost undiminished numbers. It seems more appropriate to assume that this organism dies because it is highly permeable than that it becomes permeable because it dies, but in any case the type of cell death leaves NH_3 production unimpaired. On the other hand death of cells due to heating at 60° for fifteen minutes or boiling for thirty minutes does not increase the diffusion of the electrolytes studied; nor does it interfere with the liberation of carbon dioxide: while it amost wholly inhibits the production of ammonia.

5. *a.* Dilute solutions of sodium chloride (0.0145 M and generally 0.145 M) tend to increase the permeability of the cell wall and to promote the diffusion of all the products studied,—ammonia and other alkaline substances (tables 1, 2, 3, 14, 15 and 16), carbon dioxide (tables 2, 4, 6 and 7) chlorides (tables 9 and 10) and phosphates (tables 11, 12 and 13) whether in the presence of normal or heated cells. It is interesting to note that this increase in permeability occurs in a salt solution which we know to be highly favorable to the viability of *Bact. coli*, indicating that increased permeability may be favorable rather than unfavorable to cell life. (In the case of CO_2 it must always be remembered that increased liberation of the substance may be, in part or in whole, due to increased production within the cell rather than to increased permeability.)

b. A strong solution of NaCl (1.450 M) and solutions of $CaCl_2$ of moderate strength (0.0145 M and 0.145 M) on the other hand decrease the liberation of ammonia and other alkaline substances (tables 1, 2, 3, 14, 15 and 16); but increase the liberation of acidic substances (see first part of table 2 and tables 4, 5, 6, 7 and 8). It may well be that the latter phenomenon is really only the result of the former or, in other words that these salts merely check ammonia formation and leave carbon dioxide production and diffusion unaffected. Our tests with phosphates suggest that 1.450 M NaCl may slightly increase permeability (tables 11 and 12).

c. Finally the strong calcium solution (1.450 M) shows with *Bact. coli* a sharp initial rise in titratable alkalinity (table 3)

and in titratable acidity (tables 4, 5, 6, 7, and 8) followed by a fall, which we interpret as due to a decrease in permeability leading to lysis and liberation of proteins followed by an accumulation of non-reactive films on the protein micellae or to absorption of the oppositely charged ions. An alternative explanation would be that strong $CaCl_2$ causes a type of membrane coagulation which opens the cell wall to the free passage of substances to which it is impermeable. In any case it is highly significant that the highly permeable membrane of *B. cereus* gives no such reactions,—the strong calcium solution (1.450 M) showing here only the increase of acidic substances and the decrease in ammonia manifest in weaker solution, though in more marked degree (tables 1 and 2).

6. Finally, we have been impressed throughout this and earlier work with the fact that the current assumption of an inherent antagonism between monovalent and bivalent ions may perhaps be an unnecessary one, at least so far as bacterial cells are concerned.

Very dilute sodium salts behave in one way and very strong calcium salts in another; and at a given molar concentration different effects and sometimes opposite effects may be manifest; but intermediate strengths of both salts may exert an essentially similar influence. May it not be possible to explain the observed phenomena on the assumption that dilute solutions of either Ca or Na tend to increase permeability and that strong solutions of either salt tend to increase it, the concentrations of NaCl to produce a given effect being of course much higher than the corresponding concentrations of $CaCl_2$? Such an assumption would be in accord with the work of Endler (1912) on the influence of salts upon the absorption and diffusion of dyes and with the work of many other observers.

In order to test this hypothesis we have employed the empirical test devised by Mines (1912) which indicates, as he believes, whether a suspended substance is in an emulsoid or in a suspensoid state. Heavy suspensions of *Bact. coli* were prepared in water and in various salt solutions and stored for one-half hour at 37°C. Varying amounts (best 3 drops) of cobalt-hexamin-

chloride (luteo-cobalt chloride) and of 0.1 M aluminum chloride were then added to the suspensions and they were again stored for twenty hours, at which time the amount of agglutination was determined macroscopically. According to Mines (1912) and Oliver and Barnard (1925) absence of precipitation in the cobalt solution and strong precipitation in the aluminum solution indicates the presence of emulsoids while strong agglutination in both solutions indicates the presence of suspensoids. Our tests indicated that in the presence of 0.0145 M, and 0.000145 M CaCl₂ the bacterial substances behaved as emulsoids, while in water and strong salt solutions (1.450 M NaCl and 0.00145–1.450 M CaCl₂) they reacted as suspensoids.

If such an explanation should prove to be justified a substantial simplification of our conception of salt action should result.

REFERENCES

ASHBY, W. 1924 Study of the mechanism of change in resistance of erythrocytes to hypotonic salt solution. Amer. Jour. Physiol., **68**, 585.

BROOKS, M. M. 1923a, b Studies on the permeability of living and dead cells. I. New quantitative observations on the penetration of acids into living and dead cells. II. Observations on the penetration of alkali bicarbonates into living and dead cells. Pub. Health Rep., **38**, 1449, 1470

BROOKS, M. M. 1925 Studies on the permeability of living and dead cells. V. The effects of NaHCO₃ and NH₄Cl upon the penetration into Valonia of trivalent and pentavalent arsenic at various H ion concentrations. Pub. Health Rep., **40**, 139.

BROOKS, M. M. 1926a The permeability of protoplasm to ions. Amer. Jour. Physiol., **76**, 116.

BROOKS, M. M. 1926b Studies on the permeability of living cells. VI. The penetration of certain oxidation-reduction indicators as influenced by pH; estimation of the pH of Valonia. Amer. Jour. Physiol., **76**, 360.

BROOKS, S. C. 1916 Studies on exosmosis. Amer. Jour. Bot., **9**, 483.

BROOKS, S. C. 1917 A new method of studying permeability. Bot. Gaz., **64**, 306.

COOK, S. F. 1926 The effects of certain heavy metals on respiration. Jour. Gen. Physiol., **9**, 575.

COULTER, C. B. 1924 Membrane equilibria and the electric charge of red blood cells. Jour. Gen. Physiol., **7**, 1.

DEBOER, S. 1917 The influence of the respiration on the exchange of SO₄ between corpuscles and plasma and its effect on the excretion of SO₄. Jour. Physiol., **51**, 211.

DOISY, E. A., AND EATON, E. P. 1921 The relation of the migration of ions between cells and plasma to the transport of carbon dioxide. Jour. Biol. Chem., 47, 377.

ENDLER, J. 1912 Über den Durchtritt von Salzen durch Protoplasma. Biochem. Z., 42, 440

FALK, I. S. 1923 The rôle of certain ions in bacterial physiology. A review (Studies on salt action, VII). Abst. Bacteriol., 7, 33, 87, 133.

FRIDERICIA, L. S. 1920 Exchange of chloride ions and of carbon dioxide between blood corpuscles and blood plasma. Jour. Biol. Chem., 42, 245.

GRAY, J. 1920a, b The relation of the animal cell to electrolytes. I. A physiological study of the egg of the trout. II. The adsorption of hydrogen ions by living cells. Jour. Physiol., 53, 308; 54, 68.

GRAY, J. 1921 Exosmosis from animal cells. Jour. Physiol., 55, 322.

GREEN, R. G., AND LARSON, W. P. 1922 Conductivity of bacterial cells. Jour. Inf. Dis., 30, 550.

GUILLEMIN, M., AND LARSON, W. P. 1922 The relation between the fixed and free salts of bacteria. Jour. Inf. Dis., 31, 349.

GÜRBER, A. 1895 Ueber den Einfluss der Kohlensaure auf die Vertheilung von Basen und Sauren zwischen Serum und Blutkorperchen. Jahresb Tierchem., 25, 164.

HAMBURGER, H. J. 1916 Die Permeabilitat von unter physiologischen Verhältnissen kreisenden Blutkorperchen für Kalium nach einer neuen Methode. Wien. med. Woch., 56, 519.

HARVEY, E. N. 1911 Studies on the permeability of cells. Jour. Exper. Zool., 10, 508.

HARVEY, E. N. 1913 A criticism of the indicator method of determining cell permeability for alkalies. Amer. Jour. Physiol., 31, 335.

HARVEY, E. N. 1914 The permeability of cells for acids. Internat. Z. physikchem. Biol., 1, 463.

IRWIN, M. 1923a The behavior of chlorides in the cell sap of Nitella. Jour. Gen. Physiol., 5, 427.

IRWIN, M. 1923b The penetration of dyes as influenced by hydrogen ion concentration. Jour. Gen. Physiol., 5, 727.

IRWIN, M. 1925 Accumulation of brilliant cresyl blue in the sap of living cells of Nitella in the presence of ammonia. Jour. Gen Physiol., 9, 235.

IRWIN, M. 1926 Mechanism of the accumulation of dye in Nitella on the basis of the entrance of the dye as undissociated molecules. Jour. Gen. Physiol., 9, 561.

JAUMAIN, D. 1922 Autolyse microbienne en tubes scellés Compt. rend. Soc. de biol., Par., 87, 790.

JOHNSON, I. S. C., AND GREEN, R. G. 1924 Conductivity of yeast cells. Jour. Inf. Dis., 34, 186.

LIPMAN, C. B. 1909 Toxic and antagonistic effects of salts as related to ammonification by Bacillus subtilis. Bot. Gaz., 48, 105.

LOEB, J. 1922 Proteins and the Theory of Colloidal Behavior. New York.

MACDOUGALL, F. H., AND GREEN, R. G. 1924 Theory of electrical conductance of suspensions. Jour. Inf. Dis., 34, 192.

MCCUTCHEON, M., AND LUCKE, B. 1924 The mechanism of vital staining with basic dyes. Jour. Gen. Physiol., 6, 501.

MEDES, G., AND McCLENDON, J. F. 1920 Effect of anesthetics on various cell activities. Jour. Biol Chem., **42**, 541.

MELLANBY, J., AND WOOD, C. C. 1923 The influence of carbon dioxide on the interchange of ions between the corpuscles and the serum of blood Jour. Physiol., **57**, 113.

MEYERS, V. C. 1924 Practical Chemical Analysis of Blood. St. Louis.

MINES, G. R. 1912 Der Einfluss gewisser Ionen auf die elektrische Ladung von Oberflachen und ihre Beziehung zu einigen Problemen der Kolloidchemie und Biologie Kolloid-chemische Beihefte, **3**, 191.

MUKAI, G. 1921 The action of CO_2 on salt and water distribution in the blood Jour. Physiol , **35**, 356

OLIVER, J., AND BARNARD, L 1925 The nature of the surface of the red blood cell and mechanisms of suspension stability. Amer. Jour. Physiol., **73**, 401

OSTERHOUT, W. J V 1922 Injury, Recovery and Death in Relation to Conductivity and Permeability Philadelphia

OSTERHOUT, W J V 1925 Is living protoplasm permeable to ions? Jour. Gen. Physiol , **8**, 131.

OSTERHOUT, W J V , AND DORCAS, M J. 1925 The penetration of CO_2 into living protoplasm Jour. Gen. Physiol , **9**, 255.

PETERS, A W 1908 Chemical studies on the cell and its medium Amer. Jour Physiol , **21**, 105

ROUS, P. 1925 The relative reaction within living mammalian tissues. Jour Exper Med., **41**, 379, 399, 451, 739.

SHAUGHNESSY, H J., AND FALK, I. S. 1924 The effects of some electrolytes on the buffering capacity of *Bacterium coli* Jour. Bacteriol., **9**, 559.

SHEARER, C. 1919 The action of electrolytes on the electrical conductivity of the bacterial cell and their effect on the rate of migration of these cells in an electrical field. Proc Camb. Phil Soc., **19**, 263.

SHEARER, C 1920 Studies on the action of electrolytes on bacteria Jour Hyg , **18**, 337

STILES, W., AND JORGENSON, I 1915 Studies in permeability. I. The exosmosis of electrolytes as a criterion of antagonistic ion-action. Ann. Bot , **29**, 349.

VAN SLYKE, D D 1923 The determination of chlorides in blood and tissues. Jour. Biol Chem., **58**, 523.

VAN SLYKE, D. D , AND CULLEN, G. F 1917 Studies of acidosis Jour. Biol. Chem , **30**, 289.

WIECHMANN, E. 1921 Neue Untersuchungen uber die Permeabilitat der roten Blutkorperchen. Deutsche med. Woch , **47**, 824

WINSLOW, C.-E. A., AND FALK, I. S. 1923 Studies on salt action. VIII. The influence of calcium and sodium salts at various hydrogen ion concentrations upon the viability of *Bacterium coli*. Jour. Bacteriol., **8**, 215.

WINSLOW, C.-E. A., AND WILLCOMB, G. E. 1905 Tests of a method for the direct microscopic enumeration of bacteria. Jour. Inf. Dis., Supplement, No. 1, p. 273.

ZOOND, A. 1927 The interpretation of changes in electrical resistance accompanying the death of cells. Jour. Bacteriol., **13**, 9.

THE INFLUENCE OF CARBON DIOXIDE ON BACTERIA[1]

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Carbon dioxide was known to the ancients. Its presence was perceived by Plenius near volcanoes and mineral springs, and it was called by him "spiritus letales." It was observed later by others, who confused it with "air." John Baptista Van Helmont (1577–1644), introducing the word "gas," called it "gas sylvestre." He knew that this gas could extinguish a flame and cause suffocation in animals. It is quite possible that these now well-known properties suggested its uses in more modern times for the attempted destruction of microbial life and therefore for the employment of this gas as a germicide.

Beginning with the work of Pasteur and Joubert in 1877, experimental evidence concerning the influence of carbon dioxide on bacteria has been accumulating. Various phases of the question have been investigated, and in many instances practical application of the incomplete knowledge has been made. However, careful scrutiny of the cumulative evidence of nearly fifty years of research must lead to the conclusion that the results of carbonation in any given case could not be predicted, and that further experimental data were necessary in order to avoid fallacious generalizations.

It has long been a matter of common knowledge that chlorophyl-bearing plants require carbon dioxide for their metabolism and growth. More recently the astonishing observations have been made and supported by independent investigators that CO_2 plays an important rôle in the respiration of man and the higher

[1] This paper embodies the work and results presented in the senior author's Doctorate Thesis deposited in the Library of Yale University.

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mammals, and that this gas is absolutely essential to their continued existence.

The first phase of the present studies was stimulated by certain recent claims that carbon dioxide gas, when introduced into commercial ice cream, in place of ordinary air, and incorporated in the ice cream by the usual process of freezing, exerts a definite germicidal action on the bacteria present in the frozen product.

Space in this JOURNAL does not permit of an extended historical review of the literature on various phases of the carbon dioxide question as related to bacteria. For such the reader is referred to the thesis in the Yale University Library.

The investigations reported in this paper fall under two distinct heads:

> I. A study of carbon dioxide as a possible germicidal or bacteriostatic agent
>
> II. An inquiry into the relation of carbon dioxide to growth and continued development of the bacterial cell

I. CARBON DIOXIDE AS A POSSIBLE GERMICIDAL OR BACTERIOSTATIC AGENT

This phase of the investigation was begun in 1922[2] and the immediate results reported in a monograph of the National Ice Cream Manufacturers' Association (1922). The chief purpose of this work was to determine the influence of carbon dioxide in carbonated ice cream on the viability of bacteria in the cream; but the scope of the investigation was such as to involve also experiments conducted on carbonated milk, and on ordinary laboratory media with and without varying amounts of added buffer.

a. Carbonated ice cream

This work was conducted in six different experiments on lots of ice cream prepared under ordinary commercial plant conditions. In each of these experiments the results obtained with the car-

bonated ice cream were compared with those of the ordinary commercial product, the two being prepared from the same stock cream and under the same conditions except for the use of compressed carbon dioxide gas (8 ounces per 20-gallon freezer)
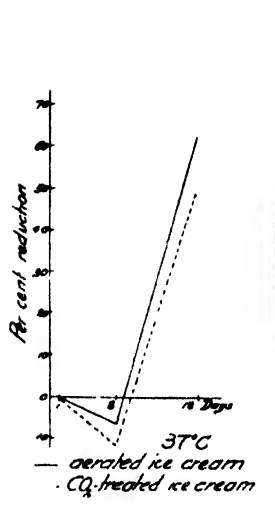
Fig 1

Fig 2

Fig. 1. Experiment 1 Uninoculated Ice Cream
Fig. 2. Experiment 2. Uninoculated Ice Cream
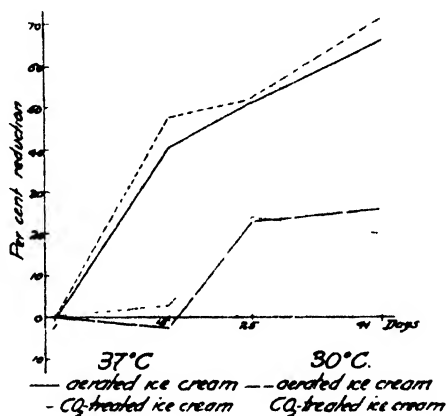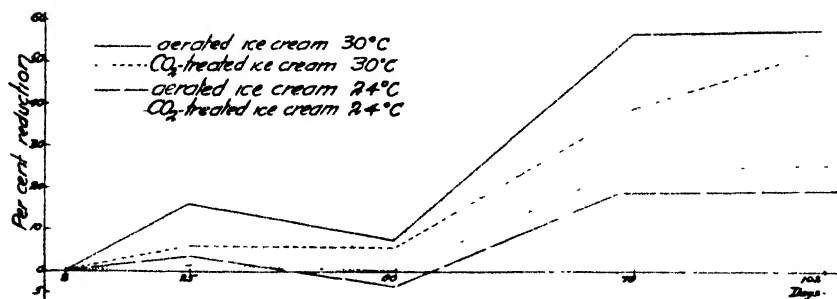
Fig. 3. Experiment 3. Ice Cream Inoculated with Streptococcus lacticus

from a cylinder in the carbonated ice cream, in place of ordinary air. The viability of the bacteria was determined by the plate counting method in plain and in lactose agar. Except in experiment 1, two sets of agar plates were employed in the tests. These

plates were incubated at two different temperatures, 24° and 30°, or 30° and 37°C.

In two of these experiments no bacterial suspensions or cultures were added to the stock mixture, and comparative viability studies were made on the natural cream mix flora. In experiments 3 and 4 *Streptococcus lacticus* was added to the stock and
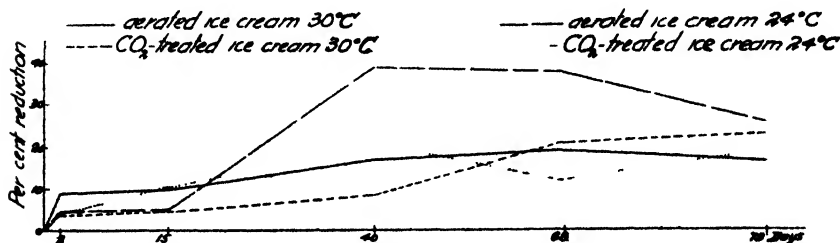
FIG. 4. EXPERIMENT 4. ICE CREAM INOCULATED WITH STREPTOCOCCUS LACTICUS
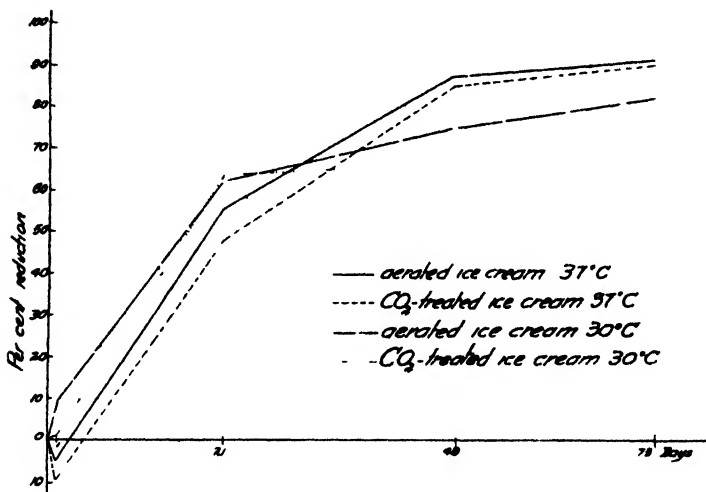
FIG. 5. EXPERIMENT 5. ICE CREAM INOCULATED WITH BACTERIUM COLI

used as test organism. Experiments 5 and 6 were conducted in very much the same manner as experiments 3 and 4, but with the use of *Bact. coli* in 5, and *B. cereus* in experiment 6.

The results of these six experiments are shown in figures 1 to 6 in six different sets of curves. For detailed statements and fig-

ures the reader is referred to the earlier publication (1922) and to the doctorate thesis of the senior author at the Yale University Library.

The results obtained in this phase of the investigation are overwhelmingly opposed to any claims that carbon dioxide in carbonated ice cream exerts a bactericidal action on the bacteria present. In experiment 6 the figures appear to favor the carbonated product. There was an apparent reduction of viable organisms of 35.6 per cent in the aerated, and of 39.6 per cent in the carbonated ice cream during the first twenty-four hours of
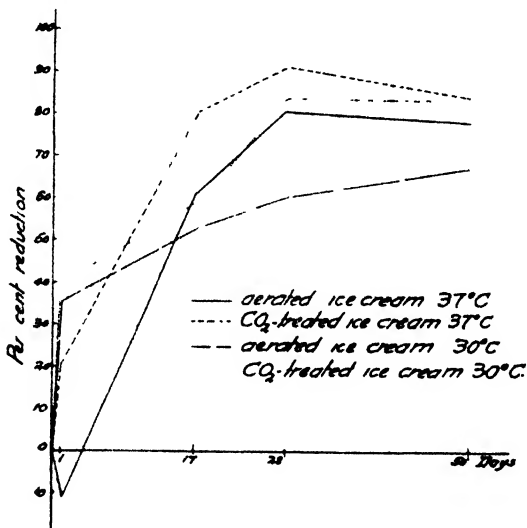
FIG. 6. EXPERIMENT 6   ICE CREAM INOCULATED WITH BACILLUS CEREUS

storage in the refrigerator room. After fifty days of holding the reductions were 67.2 and 83.1 per cent respectively.

In all of the other (five) experiments no germicidal or bacteriostatic action of carbon dioxide could be demonstrated. No marked differences in viability counts were seen, and the slight variations which are shown are on the whole on the side of the aerated, and not the CO_2-treated products. These results were borne out in the subsequent experiments on carbonated milk and laboratory media.

Winslow and his associates (1922) showed that by the use of 8 ounces of CO_2 per 20-gallon freezer of ice cream the gases in frozen product contained from 52 to 55 per cent CO_2. They were unable to increase the CO_2 content of the ice cream beyond 50 to 60 per cent of the air or gases in the product, even when as much as 28 ounces of CO_2 gas were introduced into the 20-gallon freezer. They showed further that the CO_2 of carbonated ice cream is diffused out during storage and that at the end of seven weeks storage the CO_2 content of the carbonated cream was no greater than the uncarbonated, viz., 10 cc. of CO_2 per 100 grams of ice cream.

It would appear from frequent observations that under ordinary atmospheric pressure the amount of CO_2 introduced is insufficient to materially affect the H-ion concentration, except perhaps at the time of charging and freezing, and that any increase in free H-ions, if there is any, is too small in amount and of too short duration to affect the viability of the bacteria present. Ice cream is highly buffered, and large amounts of CO_2 are necessary to effect any appreciable change in pH. The question of the influence of increased H-ion concentration, and the restraining action of a buffer will be discussed later.

b. Influence of carbon dioxide in fresh milk

Carbonic acid gas was bubbled through unpasteurized skim milk in open containers, at a gauge pressure of 2 pounds, and for a period of three minutes.

In the first experiment an initial count of 92,000 per cubic centimeter at 37°C. was obtained with both the carbonated and uncarbonated samples. At the end of 24 hours standing at 20° to 23°C. the respective plain agar plate counts were 1,340,000 and 1,020,000 per cubic centimeter of milk. On lactose agar plates kept at 30°C. for twenty-four hours the untreated milk registered 500,000,000 as compared with 1,090,000,000 for the carbonated.

In a second experiment both the carbonated and uncarbonated milk showed 129,000,000 organisms per cubic centimeter, with an initial count of 23,000. Quite similar results were obtained

in a number of other tests, and it was not possible to demonstrate any general inhibitory action of carbon dioxide.

While the viability tests revealed no influence on the total number of organisms by the CO_2, there was apparently at times some difference in the types of colonies on the plates and the general appearances of the plates prepared from the two kinds of milk. The CO_2 seemed to exert a selective action, but did not enhance the keeping quality of the milk or prevent rapid bacterial proliferation in it.

c. The influence of carbon dioxide on bacteria in pure culture in plain laboratory media

In this study the action of various concentrations of CO_2 gas was determined on 19 different bacterial species.

Carbonic acid gas is soluble in pure water, and its solubility increases directly with increase in pressure and inversely with increase in temperature. At ordinary atmospheric pressure and at room temperature water takes up sufficient CO_2 to render it distinctly acid, the degree of acidity depending on the amount of CO_2 introduced.

Larsen, Hartzell and Diehl (1918) have shown that the degree of acidity of CO_2 solutions under pressure lies between pH 3 and pH 4. They also state that the theoretical increase in hydrogen ion concentration from pH 3.6 under one atmosphere to pH 3.15 under 50 atmospheres was difficult to show experimentally. Consequently the pressure is a minor factor in increasing the acidity of CO_2 solutions beyond a certain point.

In our experience the lowest value found was pH 3.8 for distilled water, when the water was saturated with CO_2. This marked depression was made possible by the complete absence of buffering agents in solution.

Methods employed. Slant agar cultures proved to be suitable for roughly quantitative work. For a more exact study the agar plating method was used. In both instances the inoculated media were enclosed in a bell jar and the desired amounts of CO_2 gas introduced from a cylinder. A uniform pressure slightly above that of the ordinary atmosphere was maintained by means

of a mercury trough which was connected with an outlet valve of the bell jar. The uncarbonated control cultures were similarly enclosed and given the same treatment except for the added CO_2.

The carbon dioxide was determined by the sodium hydroxide absorption method in the Orsat-Lunge gas analysis apparatus fully described in bulletins 42 and 197 of the United States Department of Commerce, Bureau of Mines.

For the determination of H-ion concentration the colorimetric method was used, involving the use of buffer solutions of Sörensen as described by Clark.

TABLE 1

Showing effect of different percentages of CO_2 on H-ion concentration of plain agar medium

ORIGINAL pH OF AGAR	PER CENT CO_2 BY VOLUME	pH AFTER TWENTY-FOUR TO FORTY-EIGHT HOURS EXPOSURE
7.3–7.2	95	5.6
7.0	95	5.6–5.5
6.8–6.6	95	5.4–5.2
6.4–6.0	95	5 2–5.0
5 8–5.6	95	5.0–4.8
5.4–5.2	95	4 7–4.6
5.0–4.6	95	4 6–4.3
6.9	70–75	5.9
6.9	45–50	6.2–5.9
6.9	20–25	6.5–6.2
Water.......	95	3 8
Gelatine, pH 6.8....	95–98	5 0–4.8

The carbon dioxide gas was supplied for these, as for all other CO_2 experiments in this investigation, from an ordinary commercial CO_2 cylinder.

Changes brought about in H-ion concentration and their effect on bacterial growth and development. With the proper color indicators (in plain agar medium) placed in the closed jars with the culture tubes or plates, definite changes in H-ion concentration in these indicator tubes are immediately observable on the introduction of CO_2 into the jars. In order to determine the exact

magnitude of these changes open indicator tubes were selected which were of the same diameter as the standard stock buffer solutions containing the same indicators, which were placed, tightly plugged, in the same jars.   Changes brought about in plain agar tubes by different amounts of CO_2 are illustrated in table 1.

On exposure to an atmosphere containing 95 per cent CO_2 a final H-ion concentration of the agar corresponding to pH 5.6 to 4.3 was attained.   As ordinary agar is slightly buffered by the peptone, amino acids, etc., contained in the commercial peptone, some difference between the final pH of the agar (4.3) and pure water (3.8) must be expected.

Observations of different strains of bacteria in pure culture.   Suspensions of the respective organisms were made in sterile physiological saline solution and a 4 mm. loopful of the suspensions streaked over the surface of slant agar tubes.   The tubes were placed in bell jars which were incubated with and without the measured volumes of CO_2, as described above.   The extent to which growth took place was determined by direct macroscopic observation and by subsequent incubation in ordinary atmosphere (see column 1 in table 3).

In order to obtain more nearly quantitative data on the influence of CO_2 on bacterial viability, dilution plates of the different organisms were poured from eighteen to twenty-four hour agar slants.   These plates were enclosed in bell jars and subjected to the same treatment as the slant agar tubes heretofore described. As the data acquired by the usual colony count do not add much information to those obtained with the slant agar cultures they are not included here.   They will be found in the senior author's doctorate thesis at the Yale University Library.

The effect of varying degrees of initial H-ion concentration in the agar medium on the individual organisms studied is shown in table 2.

A close study of this table shows that different organisms reveal very marked differences in their tolerance or lack of tolerance for increased H-ion concentration of the agar medium.   For example, *Staph. aureus*, *Bact. aerogenes*, and *E. prodigiosus* possess a relatively high tolerance, while *V. cholerae* occupies the other extreme

end of the series, and *Ps. pyocyanea* and *Ps. fluorescens* occupy a middle portion.   The list of organisms is given in the order of decreasing tolerance.

None of the species are appreciably affected in the range pH 7.4 to 6.4, and little effect is exerted within the range 6.2 to 5.6.

TABLE 2

Showing the effect of increased H-ion concentration on 19 different organisms

ORGANISMS	pH OF THE AGAR MEDIUM						
	7 4-6 4	6 2-5 6	5 4-5 0	4 8	4 6	4 4	4.2
Staph. aureus	4	4	4	4	4	3	3
Bact. aerogenes .	4	4	4	4	3	3	3
E. prodigiosus .	4-3	4	4 3	3	3	3	3
M. tetragenus	4	4	4	3	3	3	0*
Bact. paratyphosum A	4	4	3-2	2	2	2	1
Bact. coli.........	4	4-3	3-2	2	2	2	1
Bact. typhosum ..	4	4 3	3 2	2	2	2	1
Bact. paratyphosum B	4	3	2	2	2	2	0*
Staph. albus (K)	4	4 3	3 2	2	2	0*	0*
B. cereus.	4	4	3 2	2	2	1	0*
Ps. fluorescens liq	4-3	4	3	2	1	0*	0*
Chr. violaceum	4	3	2	2	2	1	0
Ps. pyocyanea	4	3	2	1	1	1	0*
B. megatherium .	4-3	4 3	3-2	1	0*	0*	0*
B. subtilis ..	4	4-2	1	1	1	0	0
Sarcina lutea	4	4-3	3-1	0	0*	0*	0*
Proteus vulgaris	4	3	2-1	0*	0*	0	0
B. anthracis. . .	4	3-1	0*	0*	0*	0*	0*
Vibrio cholerae .	4 3	3-1	1-0	0	0	0	0

Note: The figures represent degrees of growth on slant agar. 0 indicates no growth, 1 slight, 2 appreciable, 3 moderate and 4 luxuriant (normal) development of the given organisms.

*The presence of the asterisk after negative symbols (0) in table indicates that growth, as a rule slight, was obtained on sub-culturing to fresh medium. The sign 0 without asterisk indicates that no growth was obtained on transfer to new medium.

At pH 4.2, however, all but the first six are entirely or almost entirely inhibited, and only the first three are able to develop to any marked degree.

As will be seen later, the organisms which were able to develop

over a wide range of H-ion concentration were also relatively resistant to the higher concentrations of carbon dioxide.

d. The influence of carbon dioxide on bacteria in buffered media

In the selection of a buffering agent for culture media serious consideration must be given to the question of harmful action of the buffer on the bacteria concerned. The agent itself and the amount used are therefore of much importance.

In these experiments di-potassium and di-sodium phosphate were employed as buffer, as it was sought to prevent appreciable acid production and no thought had to be given to over-alkalinization.

The phosphate was added to the agar medium in two different concentrations, namely 0.25 and 1.0 per cent. The reactions obtained in the two phosphate agars were as follows:

	pH
0 25 per cent di-sodium phosphate	7.1
1.0 per cent di-sodium phosphate .	7 8
Plain agar, without the phosphate	6 9

These three concentrations of phosphate were found on further observation not to be inhibitive to any of the organisms employed.

The experiments were performed as follows: The variously buffered media were slanted in the usual way in test tubes and inoculated with 4 mm. loopfuls of bacterial suspensions which were prepared from eighteen hour agar cultures. Each series was inoculated in duplicate, one being subjected to CO_2 treatment and the other held as a control. Forty-eight hours were allowed for the incubation.

Fifteen different organisms were employed. The results are presented in table 3.

In this work the different organisms were exposed to 95 to 97, 70 to 75, and 50 to 55 per cent CO_2, in tubes of plain agar containing 1.0, 0.25 per cent, and no added buffer respectively.

The table shows the bacterial development obtained with the fifteen different organisms; also the original pH and that of the different culture tubes after forty-eight hours of exposure to the

CO_2 or air. The CO_2 percentages were obtained by chemical analysis of the gas in the culture jars.

These observations must be regarded as further proof that CO_2 in itself is not antiseptic or bactericidal, and that any harmful action brought about on bacteria by the application of CO_2 is

TABLE 3

Showing the degree of growth on buffered and plain agar medium under different percentages of carbon dioxide

ORGANISMS	Per cent buffer	95 to 97 per cent			70 to 75 per cent			50 to 55 per cent			Air control		
		0	0.25	1 0	0	0 25	1 0	0	0.25	1.0	0	0 25	1.0
Bact. aerogenes ...		4	4	4	4	4	4	4	4	4	4	4	4
Bact. coli		2	4	4	4	4	4	4	4	4	4	4	4
Bact. paratyphosum A		2	3	3	3	3	3	3	4	4	4	4	4
Bact. paratyphosum B		2	3	3-4	3	4	4	3-4	4	4	4	4	4
Bact. typhosum.		2	2	3	3	3	3-4	4	4	4	4	4	4
Streptococcus pyogenes .		3	3	4	3	3-4	4	3-4	4	4	4	4	4
Staph. albus (k)		2	3	3	2-3	3	3-4	3	4	4	4	4	4
B. cereus ...		1·2	3	3-4	3	4	4	4	4	4	4	4	4
B. anthracis.		0	3	3	1	4	3-4	2	4	4	4	4	4
B. subtilis		1	3	3	2	3	3	2	3	4	4	4	4
V. cholerae		0	0	3	0	0	3	0	3	3-4	4	4	4
Isolations from ice cream													
Organism no. 1.. ...		0	0	2	0	1	2-3	0	3	3	4	4	4
Organism no. 10		3	4	4	3	4	4	4	4	4	4	4	4
Organism no. 11		3	4	4	4	4	4	4	4	4	4	4	4
pH after forty-eight hours.....		5.2	5.9	6.8	5.9	6.2	7.2	5.9-6.2	7.0	7.4	6.8	7.1	7.8
Original pH.......		6.9	7.1	7.8	6.9	7.1	7.8	6.9	7.1	7.8	6.9	7.1	7.8

Note: 0 indicates no growth, 1 slight, 2 appreciable, 3 moderate, and 4 luxuriant (normal) development.

exerted through the increased H-ion concentration of the medium exposed to the gas. When sufficient buffer is added to the medium to prevent any appreciable change in the H-ion concentration, even the most delicate of the organisms used here (*V. cholerae*) will develop in atmospheres containing as much as 70 to

75 per cent CO_2.   The more vigorous and resistant organisms, as for example, *Bact. aerogenes* and *Staph. aureus*, grow well under an atmosphere of 95 to 97 per cent CO_2, in both the plain agar and in that containing added buffer.   For some organisms, therefore, added buffer does not appear to be essential to growth, owing to their marked tolerance for free H-ions.

II. THE RELATION OF CARBON DIOXIDE TO GROWTH AND CONTINUED DEVELOPMENT OF THE BACTERIAL CELL

The foregoing experiments were concerned with high concentrations of CO_2, but it was impossible to demonstrate any injurious action on bacteria so long as material increases in H-ion concentration were prevented by the use of a proper buffering agent.

With low concentration of CO_2 (less than 10 per cent) the changes brought about in H-ion concentration of the agar medium, without added buffer, are insignificant, and very little, if indeed any, hindrance to the growth of bacteria, even the most sensitive studied here, is offered.

In nature bacteria are constantly exposed to an environment in which CO_2 is present, though at times in very small amount. For example, ordinary air contains about 0.03 per cent, animal fluids and tissues possess varying amounts of this gas, and in the soil and in decaying organic matter generally CO_2 is produced as a result of the activities of microorganisms and of plant roots.

Carbon dioxide in 10 per cent concentration has been advocated and used by various investigators (Cohen and Markle (1916), Chapin (1918), Cohen and Fleming (1918), Huddleson (1920)) in the cultivation of microaerophiles or "partial tension" organisms.   The purpose of the gas was not to supply a favorable or necessary agent as such, but as a diluent of atmospheric air, thus rendering the gaseous environment favorable, as was supposed, because of its reduced oxygen.

The following question presented itself: If carbon dioxide is not in itself harmful to bacteria, may it not be beneficial in small amounts, and perhaps necessary in the physiology of bacteria, as it is in that of the chlorophyl-bearing plants and as it has recently been shown by Henderson and others to be in human and animal

physiology? The following experiments were conducted, therefore, to determine the relation of small quantities of CO_2 to the life and growth of the bacterial cell; in other words, to study the response of bacteria in pure culture to the removal of carbon dioxide in part or in whole from the environment.

There are apparently no experimental data on record which show what the exact gaseous requirements for the bacterial cell are, and this is particularly true in so far as CO_2 is concerned.

The cell derives its main supply of carbon dioxide, if indeed such is needed, from the atmosphere in which the culture is growing and from the gas dissolved in the medium. Theoretically, the equilibrium in tension is maintained by diffusion.

a. The effect of partial removal of carbon dioxide from the gaseous environment

Slants of plain nutrient agar were inoculated in duplicate, each with a 4 mm. loopful of the bacterial suspension. In one set of tubes soda lime was placed over the lowered plugs, while the other set was used for control. Both sets were placed in a Freas vacuum oven. The air was removed from the oven with a vacuum pump which registered a vaccum on the gauge of 28 to $29\frac{1}{2}$ inches. Air was then allowed to enter the oven through a scrubbing tower containing a 33 per cent solution of sodium hydroxide and glass beads. The operation of evacuating, and refilling with CO_2-free air was repeated three times. After the final aeration incubation was allowed to proceed for from eighteen to twenty-four hours..

The two sets of tubes were thus subjected to the same treatment, aside from the use of soda lime in the one set. The soda lime may have absorbed some moisture from the medium and thus caused a certain, though insignificant, desiccation. The responses obtained with the organisms employed are given in table 4.

Only those organisms which showed impeded growth are included in this table. Of the total number of organisms tried the majority appeared not to be affected in their cultural development by the soda lime, and the growths in the two sets of tubes were practically equal.

It was assumed that only partial removal of CO_2 had been effected by this preliminary method, and that further efforts would be required to remove the small amounts of CO_2 which were held in the medium. The following additional methods were given a trial.

1. Inoculated slant agar tubes were placed inverted in a beaker containing a 20 per cent NaOH solution, and incubated. This method yielded results quite similar to those recorded in table 4.

Considerable dessication occurred in these tubes. Furthermore, it was quite clear that the enclosed volume of air was very

TABLE 4

Showing the effects of reduced carbon dioxide

Soda-lime used as an absorbent.   Slanted agar cultures employed

ORGANISMS	AIR CONTROL		SODA-LIME TUBE	
	24 hours	48 hours	24 hours	48 hours
V. cholerae	3	4	0	2
Bact. typhosum	4	4	1	3
Ps. pyocyanea	3	4	0	2
Proteus vulgaris	3	4	0	2
B. subtilis .	4	4	1	4
B. megatherium	4	4	1	4
Bact. coli .	4	4	1	3
M. tetragenus	4	4	1	4

Note: 0 indicates no growth, 1 very slight (one or two colonies on the slant), 2 appreciable, 3 moderate, and 4 luxuriant growth.

small, not over 30 to 40 cc. Partial inhibition may have been due to the combined effect of these two factors.

2. The inverted tubes in the NaOH solution were provided with glass U-tubes. One arm of the U-tube extended into the culture tube close to the agar surface, and the other communicated with the CO_2-free interior of the Freas incubator, thereby providing ample supply of air in the tube.

3. Tubes of slanted agar were enclosed in the air-tight oven. The oven was then evacuated and CO_2 free air admitted to the oven. The operation was repeated four times. The oven was finally exhausted ($29\frac{1}{2}$ inches on gauge) and allowed to remain

thus for about twenty-four hours.   The tubes of agar were then removed and quickly inoculated in duplicate.   One set was immediately incubated in a CO_2-free atmosphere, and the other under ordinary air conditions.

The results obtained were essentially the same as those described in table 4.

4. A combination of low temperature and vacuum was next applied in such a way as to prevent any absorption of CO_2 by the medium.   A freezing mixture of ice and sodium chloride was prepared.   Tubes of heated and unsolidified agar were immersed in this mixture in slanted position and held in a vacuum for six hours. Immediately after inoculation the agar slants were returned to the freezing mixture in vacuum.

In spite of the lessened penetration of the CO_2 gas by rapid cooling of the agar, and the delay in bacterial growth at this low temperature, some of the gas was apparently still held in the agar medium, for on subsequent incubation at the optimum temperature in a CO_2-free atmosphere pronounced growth was obtained with several of the organisms.   In the case of the more susceptible ones only a partial inhibition was effected.   The results were as a whole rather suggestive, but could not be accepted as proof for or against a CO_2 requirement hypothesis.

The subsequent experiments were conducted on a more distinctly quantitative basis, involving the use of the Petri dish principle.

Experiments involving the principle of quantitative dilution and plating in Petri dishes.   Deep, uniform Petri dishes were selected and given a required amount of inoculum with measured amounts of agar (12.5 or 15 cc.).   The nutrient agar was prepared with Difco bacto-peptone and Liebig's meat extract, the standard method of preparation of the American Public Health Association being followed.

A 33 per cent solution of NaOH was employed at the outset as a CO_2 absorbent.   This solution was held in small Petri (Esmarck) dishes which rested on the inner surface of the cover of the inoculated inverted Petri plates during the incubation. This method allowed very rapid CO_2 absorption from the atmos-

phere and was expected to remove at least a goodly portion of this gas from the agar itself.

The organisms employed were laboratory stock strains of the various types and species. The agar plates were poured from quantitative dilution bottles, three different bottles being used for each organism. The first of these bottles (a) was inoculated with the amount of an eighteen-to-twenty-four-hour agar culture which in preliminary trials was found to give a suspension of the desired density for the given organism. One cubic centimeter of this suspension (a) was transferred to the second dilution bottle (b) containing 99 cc. water, and after shaking 25 times 1 cc. was transferred from this to dilution bottle (c).

The plates were poured with 1.0 and 0.1 cc. quantities of suspension b and with 1.0 cc. of dilution c. In each of the experiments the plates were poured in duplicate and in some instances in triplicate.

One set of the plates was incubated in an ordinary incubator, and the other in a Freas vacuum oven in which a 33 per cent solution of NaOH and boiled water were enclosed. A slow stream of CO_2-free air obtained by use of the NaOH scrubbing tower already described was drawn through the Freas incubator for a period of thirty minutes. The air issuing from the incubator was drawn through a column of barium hydroxide to test the effectiveness of the CO_2 removal system.

Incubation was as a rule conducted for from eighteen to twenty-four hours, and after a brief observation for growth, was continued for an additional period of twenty-four hours. The temperature of incubation was the optimum for the given organisms.

Complete growth inhibition was obtained by this method with the following organisms: *Bact. typhosum* (7 different strains, *Bact. para-typhosum* A and B, *Bact. coli*, *Bact. aerogenes*, *B. cereus*, *Proteus vulgaris*, *Chr. violaceum*, *V. cholerae*, *Staph. aureus* (one old and one new strain), *Ps. pyocyanea* (2 strains), *E. prodigiosus*, *Sarcina lutea* and *L. acidophilus*.

One objectionable feature was recognized in the above method. While complete inhibition of growth was readily obtained, the portion of the agar immediately over the plate containing the

concentrated NaOH solution suffered pronounced dessication, which could not be entirely eliminated by the use of weaker NaOH solution. The inhibition of bacterial growth may have been caused, at least partly, by the drying.

An attemp was made to determine the effect of drying without simultaneous absorption of carbon dioxide, by the three following methods:

1. Use of the sodium hydroxide in ordinary air and in an ordinary atmosphere containing added carbon dioxide. No inhibition of growth was obtained in either of the plates, in spite of the fact that the portion of the agar immediately over the NaOH solution was apparently completely dry. Numerous colonies developed in the area outside of the thoroughly dried zone.

2. The use of dry calcium chloride. The method is the same as the one just described, except that dry $CaCl_2$ was placed in the Esmarck dish in place of the NaOH solution. Triplicate plates were employed in each experiment. The same degree and area of drying in the agar plates (20 to 25 mm. in diameter) were established here as in the above method. One of these plates was kept in the ordinary atmosphere, the second in a CO_2-free gaseous environment, and the third in an ordinary atmosphere to which CO_2 was added from a cylinder.

The plates which were incubated in the CO_2-free atmosphere gave no evidence of growth, while those which were kept in ordinary air and under increased CO_2 showed abundant bacterial development, the growths extending to the very edge of the desiccated area.

3. By the removal of the sodium hydroxide solution from the inverted Petri dishes, after preliminary incubation, and subsequent incubation for twenty-four or thirty hours in the ordinary incubator. Growth was invariably obtained on the plates, though the numbers of colonies obtained were never as high as those observed on the ordinary air control plates.

This experiment showed that growth will occur as soon as CO_2 is admitted to the plates, in spite of the additional drying of the agar during the second incubation.

The drying and removal of CO_2 occurred at the same time, and it must be assumed that some injury to individual bacterial cells was caused through these two combined influences or by the drying alone. It appeared, therefore, that further evidence of a direct nature was highly desirable. The following additional procedures were indicated as probable means of solution of the question.

a. Removal of carbon dioxide by an absorbent which is not a dehydrating agent.

b. Preparation and employment of media which are entirely free from dissolved and combined carbon dioxide.

For attaining the first of these objects, the hydroxides of calcium, barium, and magnesium were looked upon as possibilities. Calcium and barium hydrate solutions, while quite efficient as absorbents, were not wholly satisfactory. The film of carbonate which developed on the surface reduced their absorptive capacity. Magnesium hydroxide was eliminated early.

Powdered calcium hydrate proved to be well adapted for the purpose. It may absorb a small amount of moisture when thoroughly dry, but when its attraction for moisture is satisfied, it serves as an excellent absorbent of CO_2.

For the preparation of a medium which is free from carbon dioxide, extreme measures were soon found to be necessary for the removal of the last traces of this agent.

In the first place it was important to work with a carbohydrate-free medium, especially when gas-producing organisms like *Bact. aerogenes* were used. Such a medium was prepared by inoculating the broth used for the agar with *Bact. coli*, incubating twelve to fourteen hours at 37°C. and removing the turbidity caused by this organism through a coarse porcelain filter.

Furthermore, it seemed desirable to have a medium sufficiently low in pH to liberate carbonic acid from its combination with bases (potassium, sodium, calcium, etc.) and to lessen its absorption by the medium.

For the study of the paratyphoid-enteritidis group of organisms which proved in our experience to be the most difficult to inhibit

the reaction of the sugar-free agar was adjusted to pH 5.5.   This H-ion concentration was found not to be inhibitive, or at best only to a very slight degree.

By the use of such agar many of the more resistant organisms were completely inhibited.   There remained a few, however, which showed a reduction of 75 to 95 per cent, but never complete inhibition.   In this last division belong *Bact. typhi-murium* II, *Bact. paratyphosum* C (Kral Strain) and *Bact. aerogenes*.   Further refinement of technique was therefore necessary before any definite conclusion regarding CO_2 requirements of bacteria generally could be arrived at.

A procedure was sought next whereby absorption of CO_2 by the medium could be prevented.   For this purpose freshly prepared sugar-free agar having a reaction of pH 5.5 was, on removal from the autoclave, placed in a 50° to 55°C. water bath and bath and tubes put into a Freas air-tight oven. CO_2-free air was passed over the cooling agar, the oven being maintained at a partial vacuum (5 inches on gauge).   The dilution bottles were also freshly prepared and sterilized, and were used immediately upon cooling to 30° to 35°C.

The cells of the most resistant organisms were washed three times in freshly prepared and sterilized saline solution.   The desired dilutions of the washed cells were made rapidly and the agar plates prepared with the CO_2-free agar.   The plates were allowed to cool in the Freas oven while CO_2-free air was drawn through the system.   As soon as the plates were cooled they were removed from the oven, inverted and a dish of calcium hydrate paste placed in them.   The inoculated Petri dishes were then returned to the Freas incubator and CO_2-free air passed through the tightly sealed incubator for thirty minutes.   After a definite interval CO_2-free air was again conducted through the oven, as an added precaution.   Fairly uniform atmospheric pressure was maintained by closing the stopcock nearest to the vacuum pump and leaving the connection with the scrubbing tower open.

The $Ca(OH)_2$ paste was prepared by placing from 5 to 10 grams of dry calcium hydrate in a small dish and adding sufficient water

to moisten the powder completely.   In preliminary tests it was clearly shown that Ca(OH)₂ does not cause dehydration of an agar medium exposed to it.   In fact, there was a slight increase in the weight of the agar plate, due evidently to absorption of moisture from the hydrate by the agar.

Only the organisms which could not be inhibited by the foregoing methods were subjected to the washing process and to the various other steps employed in this extreme method, namely, *Bact. aerogenes*, *Bact. typhi-murium* II, *Bact. enteritidis* and *Bact. paratyphosum* C (Kral Strain).   Complete inhibition of this group of organisms was obtained by this combination of measures.

By the use then of the more simple and of these extreme measures all organisms (strains and species) employed thus far in the investigation have been shown to require carbon dioxide for their growth and continued existence.   The minimal amounts of CO_2 required by the different organisms varied with the species and at times even with individual strains, as was shown by the responses of the various organisms to the lesser or greater reduction of CO_2 in the environment.   On this basis they may be regarded as falling into several distinct classes or divisions, to which more detailed reference will be made later in this paper. A summary of all of the organisms employed and the results of the quantitative study will be found in table 5.

Carbon dioxide and anaerobic bacteria.   A study of similar phenomena among the anaerobes required several modifications of the previous technique.   Quantitative tests could not be made on account of the difficulty involved in such studies.   Four well-known anaerobes were employed in these tests, *Cl. sporogenes*, *Cl. welchii*, *Cl. tetani* and *Cl. putrificum*.

Glucose agar plates (1 per cent glucose, 2 per cent agar, pH 7.2) were poured and allowed to cool in a CO_2-free Freas oven. The plates were then streaked with a bent glass rod from the liquid portion of young egg-meat cultures.   The inoculated plates were introduced into jars and the air removed by the hydrogen displacement method.   The incubation time was twenty-four to forty-eight hours, except with *Cl. putrificum* where much longer time was allowed.

TABLE 3

Showing the different responses of the organisms employed to different degrees of CO₂ removal, and groupings according to these differences

Group 1.   Strains inhibited by the simple process of removal of CO_2 from the atmosphere (41 strains)

ORGANISMS	COLONIES ON AGAR PLATES		ORGANISM	COLONIES ON AGAR PLATES	
	In ordinary air	In CO₂-free air		In ordinary air	In CO₂-free air
V. cholerae (R.)	102	00	Bact. typhosum (O)	735	00
V. cholerae (Y)	159	00	Bact. typhosum (C)	400	00
V. cholerae (5)	46	00	Bact. typhosum (G)	500	00
V. metschnikovi	40	00	Bact. typhosum (D)	425	00
Zopfius zenkeri	95	00	Bact. typhosum (N)	380	00
Proteus vulgaris	475	00	Bact. typhosum (L)	251	00
Bact. abortus (Wo)	600	00	Bact. typhosum (S)	145	00
Bact. abortus (Wn)	467	00	Bact. typhosum (Kr. 79)	245	00
Bact. abortus	750	00	Bact. pullorum (R)	547	00
L. acidophilus (Sc)	Crowded	00	Bact. pullorum (B 16)	348	00
L. acidophilus (R-1)	4,500	00	Bact. pullorum (R 23)	258	00
L. acidophilus (1B)	1,575	00	Bact. pullorum (Wh)	680	00
L. acidophilus (No. 20)	4,450	00	C. diphtheriae (No. 8)	Crowded	00
L. acidophilus (LB)	Crowded	00	C. diphtheriae (No. 4013)	Crowded	00
L. acidophilus (GV1)	Crowded	00	B. anthracis	39	00
Bact. typhosum (Hopk.)	249	00	M. tuberculosis (W)	Growth	00
Bact. typhosum (19)	217	00	M. tuberculosis (Chr)	Growth	00
Bact. typhosum (Raw)	567	00	Proteus hydrophilus (5 strains)	Crowded	00
Bact. typhosum (R)	739	00			

Group 2. Strains inhibited by combined influence of removal of CO_2 from atmosphere and the use of freshly prepared medium. $Ca(OH)_2$ present as absorbent (15 strains)

Strep. pyogenes.	350	00	Cl. putrificum	Surface growth	00
Staph. aureus (NR)	175	00	Cl. sporogenes	Surface growth	00
Staph. aureus (NB)	231	00	Cl. welchii	Surface growth	00
Chr. violaceum	188	00	Cl. tetani	Surface growth	00
Ps. pyocyanea (NG)	95	00		Surface growth	
Ps. pyocyanea (NP)	112	00			
Ps. pyocyanea (NR)	101	00	Bact. gallinarum (c)	234	00
Bact. coli (mouse)	3,000	00	B. subtilis	75	00

Group 3. Conditions same as for group 2, except for the employment of sugar-free (coli fermented) agar, having a H-ion concentration of pH 6.8 (31 strains)

Sarcina lutea	101	00	Bact. coli (460)	276	00
Sarcina aurantiaca	117	00	Bact. coli (434)	4,080	00
M. tetragenus	139	00	Bact. coli (465)	301	00
E. prodigiosus (R)	385	00	Bact. coli (chicken)	2,560	00
E. prodigiosus (W)	165	00	Bact. enteritidis (Gärtner)	112	00
Ps. pyocyanea (O)	183	00	Enc. pneumoniae	Crowded	00
Bact. cloacae	Crowded	00	Bact. dysenteria	2,075	00
Bact. coli (horse)	280	00	B. subtilis (Shelton)	85	00
Bact. coli (rat)	Crowded	00	B. prausnitzii	Crowded	00
Bact. coli (H 355)	345	00	B. mycodes	67	00
Bact. coli (H376)	251	00	B. mycodes (am.)	37	00
Bact. coli (sheep)	452	00	B. megatherium	121	00
Bact. coli (442)	208	00	B. cereus (cells washed)	45	00
Bact. coli (365)	181	00	B. mesentericus (cells washed)	175	00
Bact. coli (436)	325	00	B. mesentericus (am.) (cells washed)	101	00
Bact. coli (446)	516	00			

TABLE 5—*Continued*

ORGANISMS	COLONIES ON AGAR PLATES — In ordinary air	In CO₂-free air
Staph. albus.....	188	00
Staph. aureus (n)	175	00
Staph. aureus (O).....	137	00
Ps. fluorescens liq........	95	00
Ps. fluorescens non. liq. . . .	113	00
Proteus mirabilis	157	00
Bact. coli (monkey).....	1,664	00
Bact. coli (401)..... .	452	00
Bact. coli (cow)......	crowded	00

Group 4. Conditions same as for group 2, except for the employment of sugar-free agar having H-ion concentration of pH 5.5 (17 strains)

ORGANISM	COLONIES ON AGAR PLATES — In ordinary air	In CO₂-free air
Bact. paratyphosum A .	123	00
Bact. paratyphosum B ...	196	00
Bact. typhi-murium II .	111	00
Bact. abortivo-equinum .	101	00
Bact. anatum (C5)	567	00
Bact. anatum (C9) .	130	00
Bact. aertryke (Can. IV) .	659	00
Bact. aertryke (Can. V) . . .	184	00

Group 5. Conditions same as for group 4, except that the bacterial cells were washed with CO₂-free saline solution (5 strains)

Organism	In ordinary air	In CO₂-free air
Bact. aerogenes (S)	270	00
Bact. aerogenes (F)	102	00
Bact. aerogenes (Sl)......	186	00
Bact. aerogenes (G)	90	00
Bact. paratyphosum C (Kr.)	115	00

The appearance of visible surface colonies was taken as a criterion of growth.   There was little difficulty in obtaining surface growth under the usual conditions, when, however, the agar was kept free from CO_2 by inverting the plates over a dish of calcium hydroxide, there was no apparent growth, providing clumps of solid egg-meat medium had not been carried over to the agar.

Cl. putrificum was very slow to develop, and as a rule the plates and large agar tubes employed remained free from visible growth under all conditions for from four to five days.   When carbon dioxide (1 to 3 per cent) was introduced into the jar growth was obtained with considerable frequency, while in the absence of CO_2 no colonies appeared in from fifteen to twenty days and in only one instance was there unmistakable evidence of bacterial development after twenty days.   The results with *Cl. putrificum* were as follows:

In six trials involving the use of 36 plates and the direct hydrogen displacement method 21 plates showed growth.

In the same number of trials with the H displacement method and added CO_2, colonies appeared in 28 plates.

Under the apparently CO_2-free conditions growth was obtained on only one out of the 36 plates.

Mycobacterium tuberculosis.   Two strains of the tubercle bacillus were employed.   One (a) was obtained from a near-by sanatorium and the other from a local tuberculosis hospital.   The latter organism (b) was designated as the Christy strain.   Both were of human origin and grew slowly.

The experiments with strain (a) were conducted on Petroff's medium containing gentian violet, 1:15,000.

The slants for air control were inoculated and sealed with paraffin.   The seal was perforated with four or five pin holes, as recommended by Novy and Soule (1925).   The tubes were incubated at 37°C. for from three to four weeks.   Out of a total of 18 tubes used 15 showed growth in the form of minute colonies which were surrounded by decolorized areas.

The same number of tubes were incubated in a CO_2-free oven for the same length of time and at the same temperature.   Only one of the 18 slants gave any indication of bacterial development.

The experiments with the Christy strain were carried out largely on alkaline glycerol beef infusion agar, as described by Novy and Soule (1925). Large culture tubes (20 x 150 mm.) were employed. The CO_2-free agar tubes were prepared by placing them (melted) in the Freas oven and passing CO_2-free air through the incubator while the agar was solidifying in the sloped form.

The glycerol-egg-medium of Lubenau was also employed. Large slants were prepared by ordinary inspissation. They were tested for sterility by subsequent incubation at 37°C. for six days. The tubes received 3 cc. of glycerol beef infusion peptone broth to avoid drying.

The inoculations were made by means of a platinum loop, the inoculum being spread as evenly as possible over the agar or egg medium surface.

The results were not wholly satisfactory, owing to contamination by moulds.

Twelve slants of the glycerol agar were enclosed in ordinary air under a bell jar. Four of these showed contamination by moulds, two no growth, and six developed small separate areas of growth in twenty-eight days

In a CO_2-free atmosphere none of the ten tubes of glycerol agar and of the eight tubes of Lubenau's medium revealed any evidence of growth.

Of the ten air control tubes of Lubenau's medium, growth was observed in three, four appeared sterile and three developed mould. The absence of growth in the four tubes may have been due to too light inoculation, in spite of the precautions taken.

All of the tubes held in the CO_2-free incubator remained free from visible growth.

The results of typical experiments on all of the organisms used in this work are summarized in table 5. These are of profound significance and were in part unexpected. In spite of the wide diversity of the different organisms studied, in morphology and in cultural and biochemical properties, CO_2 appears to be essential to the vital activities and cell functions of all of the 109 strains. The minimal requirement of the different organisms or different

strains varies within rather wide limits.   For purposes of ready comparison they may be tentatively divided into more or less distinct groups, on this basis, as is shown in the table.   It is clearly recognized, however, that due to this variation the position of an organism may be shifted in the groups.   For example, certain old laboratory strains were less sensitive to the removal of CO_2 than the freshly isolated strains.   *Staph. aureus* and *Ps. pyocyanea* serve as illustrations.   Hence, the placing of freshly

TABLE 6

Giving the number of colonies obtained in ordinary air and in air with increased CO_2 content (0 3 to 1.0 per cent)

ORGANISMS	AIR CONTROL	AIR WITH 0 3 TO 1 0 1 ER CENT CO_2
Bact. gallinarum	60	118
Bact aerogenes	270	383
Bact coli (cow) .	275	311
Bact. coli (horse)	45	65
Bact. dysenteriae	229	336
Bact pneumoniae	141	181
Bact. typhosum (R)	2,418	3,162
Bact. cloacae .	102	186
Bact. coli (monkey)	58	145
Pr mirabilis	932	1,036
Staph. albus	107	187
V cholerae .	76	120
L. acidophilus (GV9)	103	258
L. acidophilus (G6)	106	250
L. acidophilus (Sc)	182	336
L. acidophilus (R-1)	12	53

Note: The figures in columns 2 and 3 represent colony counts on agar plates of the same dilution.

isolated strains in group 2, and of laboratory stock strains of the same species in groups 3 and 4 should not be surprising.

It would seem that the function of the CO_2 is not that of an added stimulus, but that it is concerned with the very existence of the cell, since incubation without CO_2 for from twenty-four to forty-eight hours reduces the number of colonies developing subsequently on the plate and often results in actual killing of

the organisms.   Examples of this devitalizing influence are found in *Bact. abortus*, *Bact. typhosum*, *V. cholerae*, *B. subtilis*, and *L. acidophilus*.

Responses to slightly increased amounts of CO_2.   While all of the organisms studied grew under ordinary atmospheric conditions (0.03 per cent CO_2) it was of interest to determine to what extent increased amounts of the gas would facilitate growth.

TABLE 7

Showing the favorable influence of increased carbon dioxide on the growth of L. acidophilus

AIR CONTROL	25 TO 30 PER CENT CO_2
Experiment I. Strain 4 B	
26,000,000	58,000,000
23,000,000	60,000,000
Average.......24,500,000	59,000,000
Experiment II. Strain 4 B	
12,000,000	53,000,000
Experiment III. Strain 4 B	
20,000,000	75,000,000
28,000,000	78,000,000
Average......29,000,000	76,500,000
Experiment IV. Strain R-1-1	
19,000,000	65,000,000

Note: Platings were made from stock milk cultures.

In the following experiment two sets of plates were inoculated with the given organisms and placed under bell jars.   One of these sets was exposed to the ordinary air in the jar, and the other to an additional 0.3 to 1.0 per cent CO_2 introduced into the jar from a CO_2 cylinder.   Ordinary plate counts were made after fifteen to eighteen hours incubation.   The results are summarized in table 6.

Pronounced tendencies to more abundant development in the presence of *increased* amounts of CO_2 were exhibited by each of the 16 organisms employed here.   Whether all bacteria give the same response to *added* CO_2 requires further study, but these results would indicate that such favorable action of increased CO_2 on growth is, to say the least, quite general and is especially noticeable and most easily demonstrated with recently isolated strains.

This observation has an important bearing on the problem of isolation and of quantitative determination of bacteria which under ordinary conditions are slow to develop on even the choicest media.   *L. acidophilus* serves as an excellent example of this type of organisms.   It was among those which suffered most readily when subjected to partial CO_2 deprivation.

As may be seen in table 7, *L. acidophilus* requires for its best development more CO_2 than is contained in ordinary air.   Furthermore, the colonies which grew under increased CO_2 environment were larger and more typical than when the plates were exposed to ordinary atmosphere (0.03 per cent CO_2).   By the method of increased CO_2 application, the incubation period could be considerably shortened.   Later observations showed that different strains of *L. acidophilus* may show a marked quantitative difference in their CO_2 needs.

Attempts were also made to induce certain atypical laboratory stock strains to produce more nearly typical agar colonies.

The amount of CO_2 employed here was probably greater than was necessary.   Further experiments must be conducted to determine the optimum CO_2 requirements.

The principle of CO_2 requirement has been now embodied in the standard procedure of this laboratory (described by Kulp (1926)) to determine the viability of *L. acidophilus* cultures and commercial products, with very gratifying results.

It is used also by McAlpine and Slanetz in the Storrs laboratory in certain studies and viability determinations of *Bact abortus*, and by Theobald Smith (1926) in his isolation work with *Bact. abortus.*

It is obvious that the magnitude of influence of increased CO_2

is not fully expressed by the counts given here. The CO_2 concentrations are those existing at the beginning of the experiments, and no measures were adopted to maintain the original concentration, not only of CO_2, but of O_2 as well. Furthermore, the CO_2 in the air control jars was increased as growth appeared.

The experimental evidence shows that carbon dioxide in greater than the minimal amounts absolutely required, enables larger numbers of bacterial cells to grow and fosters earlier initial development and a shorter incubation period.

GENERAL DISCUSSION

The inquiry into the effects of carbon dioxide on bacteria was begun as a part of the present investigation at a time of unusual interest in the question.

The results of examination of six different lots of ice cream are shown in figures 1 to 6, and from data presented here the conclusion seems warranted that carbonation at the usual atmospheric pressure exerts very little, if indeed any, inhibitive action on the organisms originally present in the ice cream, and upon different species introduced into the ice cream as test organisms. Quite similar results were obtained with milk as regards both the natural milk flora and the test organisms used. *Strept. lacticus*, *Bact. coli*, *Bact. typhosum*, and *Bact. paratyphosum* A and B remained apparently unaffected by an atomosphere of 95 to 97 per cent CO_2.

Pure culture studies in nutrient agar, with and without added phosphate as buffer demonstrated that whatever harmful action may be exerted by CO_2 is the result of increased H-ion concentration, and is not due to the CO_2 per se. In the unbuffered agar marked inhibition of growth of a number of species was caused by only moderate increases in the H-ion concentration. Color production also was interfered with in certain instances. Some organisms were killed when the pH of the medium reached 5.6 to 5.2. Among such examples were certain strains which were isolated from carbonated ice cream.

The addition of sufficient phosphate buffer to prevent marked changes in H-ion concentration during carbonation resulted in

continued and unhindered growth. Hence any medium which contains considerable amounts of buffering agents, as for example, ice cream, milk, etc., will require the presence of correspondingly large quantities of CO_2 to cause increased keeping properties through the influence of CO_2.

Hoffman (1906) showed that milk could not be sterilized by the use of 50 atmospheres of CO_2, though some reduction in the number of viable organisms was obtained. Van Slyke and Bosworth (1906) claimed that carbonation of milk without added pressure did not exercise any preservative action. Lactic fermentation was slightly delayed, however. Hunziker (1924) and Prucha and his associates (1925) have made observations similar to ours concerning carbonation of cream and butter.

The results of our experiments are in accord with those of Prucha, Brannon and Ambrose (1922, 1925) and other investigators, but are somewhat at variance with the claims of Prescott and Parker (1924).

That it is possible at least partially to inhibit or reduce bacterial development by the use of CO_2 was clearly shown by Buchner (1885), Leone (1886), Hochstetter (1887), Frankel (1889) and others.

The results obtained by different investigators have been quite variable, and attempts to reconcile the different views have been few. D'Arsonval, in his controversy with Sabrazes and Bazin (1893), maintained that the differences are due to experimental technic, and that generalizations regarding the ultimate effects of carbon dioxide on bacteria cannot be stated a priori.

Fraenkel (1889) showed that a gelatin medium changes from alkaline to acid reaction, on treatment with CO_2 gas. The changes were not regarded as very pronounced and were supposedly of no consequence insofar as bacterial development was concerned.

Such changes are now known, by the more exact pH determination methods, to be quite appreciable (see table 1). That acidity (free H-ions) plays a very prominent rôle in the unfavorable influence brought about on bacteria in carbonated beverages or soft drinks was shown by Koser and Skinner (1922).

Briefly then, it seems evident that in order to exert any inhibitive or bactericidal action, CO_2 must be present in sufficient amounts to overcome any buffering agents that may be present and to increase the H-ion concentration to the point where it becomes growth-inhibiting or actually germicidal.

The present study of the influence of small amounts of carbon dioxide on the growth and activities of bacteria has clearly shown that CO_2, instead of being regarded purely as a waste product, is beneficial, and it appears from the observations made thus far to be essential to the continued existence of the bacterial cell.

Previous investigators have made isolated observations which would have long ago led to the above conclusions, if they had been followed up at length and proper interpretations had been made.

Szpilman (1880) claimed to have demonstrated motility by *B. anthracis* when a slow stream of CO_2 was passed through a drop culture of the organism. Grossmann and Mayerhausen (1881) made similar observations on other organisms. Nowak (1908) obtained satisfactory growth of *Bact. abortus* under two atmospheres, and under reduced oxygen tension. That this organism, which is microaerophilic, should be able to develop under these two apparently reversed conditions seemed to Nowak on the surface contradictory. The tandem method (use of *B. subtilis*) of Nowak (1908) is a practical one, as is amply proven by its varied application in the primary isolation of the gonococcus (Wherry and Oliver (1916), Chapin (1918)) and of the meningococcus (Cohen and Markle (1916)).

The favorable influence of *B. subtilis* and similar organisms employed in the tandem method has been generally regarded as being due to partial removal of oxygen from the closed jar and thus to the creation of a more favorable (non-inhibitive) gaseous environment, or to a greater moisture content, since desiccation is prevented in the closed system (St. John (1919) and Torrey and Buckell (1921)).

Some workers (Gates (1919) and Kohman (1919)) ascribed the benefits of the above methods to a delicate adjustment of H-ion concentration by the creation of a suitable buffer system.

The favorable action of *B. subtilis* and of added carbon dioxide on microaerophiles may now be regarded as being due to the satisfying of a definite CO_2 requirement which ordinary air is incapable of meeting.

Vinogradski (1899) showed that the autotrophic nitrifying soil bacteria require CO_2. His observations were confirmed by Godlewski (1892, 1895, 1896). Bonazzi (1921) and Gowda (1924). The same thing has been demonstrated for *Spirophyllum ferrugineum* (Ellis) by Lieske (1911), and more recently for *Thiobacillus thiooxidans* by Waksman and Starkey (1922, 1923) and Starkey (1925).

This conception in the domain of general bacteriology has been more gradual and more recent in its development. Wherry and Ervin (1918) held that *M. tuberculosis* requires CO_2 for development. Corper (1921), Rockwell and Highberger (1926) made similar observations. Recent experiments in the Yale and Storrs Station Laboratories have shown without question the need of CO_2 by *Bact. abortus*. Th. Smith (1924, 1926) showed that CO_2 is highly favorable in the cultivation of the Bang bacillus, and especially in the primary isolation of the parasitic strains of this organism.

Rockwell (1923), in his experiments with nine different species of bacteria which were grown in mixtures of various gases, concluded that carbon dioxide is necessary for these species. Unfortunately the experimental technique was such as to leave considerable doubt as to the merits of the claim.

The conclusions of Wherry and Ervin (1918), and of Corper, et al, were disputed by Novy (1925) who claimed that the failure of *M. tuberculosis* to develop in the experiments conducted by these workers may be ascribed to desiccation of the medium, and not to the absence of CO_2.

Rockwell (1924) believed that anaerobes are stimulated in their development by carbon dioxide, and on this as a basis advised the use of sodium bicarbonate phosphate pyrogallol mixture in place of the usual NaOH pyrogallic acid combination.

There is a fundamental difference between the theory that CO_2

merely stimulates cellular activity and the conception that bacteria have an absolute CO_2 requirement.

The latter conception can be proved only by the removal of CO_2 from the environment. The chief aim of the present work was to determine by direct procedure whether bacteria cease to grow when completely deprived of this agent, under conditions otherwise highly favorable.

The conclusion that all of the different organisms studied require CO_2 was arrived at by the use of what must be considered an adequate system of controls. Thorough checks on experiments which involved the use of a dehydrating agent like strong NaOH solution appeared especially important.

Over one hundred different organisms (109) representing widely different families and genera were employed. Not all were inhibited in their growth with the same degree of ease. For example, the following were very readily arrested: *Proteus vulgaris, C. diphtheriae, Bact. abortus, Bact. pullorum, Bact. gallinarium, Bact. pneumoniae* and *Bact. typhosum*. Others required very exacting technique to reduce the CO_2 content of the atmosphere and the medium below the growth minimum. To this group belong *Bact. paratyphosum* A, B and C, *Bact. anatum, Bact. typhi-murium* and *Bact. aerogenes*.

Some of the organisms which were easily inhibited by the removal of CO_2 in turn grew much better in an atmosphere containing added CO_2 (1 to 10 per cent) than in ordinary atmosphere (0.03 per cent). *Bact. abortus* and *L. acidophilus* were good examples of this group.

There is some evidence that the CO_2 requirements of members of the same species may vary, and that the same strains may have different requirements at different times. Further evidence is needed, however, to arrive at definite conclusions regarding this phase.

SUMMARY

The following conclusions seem warranted, as the result of the present investigation.

The carbonation of ice cream under ordinary atmospheric

pressure is a negligible factor in the reduction of numbers of viable bacteria in the stored product.

CO_2 treatment of raw milk at the same pressure likewise results in little or no bacteria-inhibiting or destroying action.

Pure culture studies with different bacteria showed a striking parallelism between bacteriostatic or bactericidal action and H-ion concentration, and it is quite evident that any harmful action brought about on bacteria by CO_2 is due to increased H-ion concentration. By sufficiently buffering the medium increase of H-ion concentration by the CO_2 may be prevented or so influenced as not to reach a point at which bacteria are unfavorably affected; and to demonstrate inhibition correspondingly larger amounts of CO_2 are required.

The removal of carbon dioxide from an environment which is otherwise favorable to development results in complete cessation of bacterial growth. This inhibition is easily accomplished with some organisms, while for others a very exacting technique is required. Small amounts of CO_2 added to the natural atmospheric environment (0.03 per cent) may and often do stimulate bacterial action and increased growth, as for example with *Bact. abortus* and *L. acidophilus.*

Anaerobic organisms also appear to require CO_2 for their development and continued existence.

From the results obtained in this investigation with more than 100 different organisms representing various families and genera the conclusion is forced upon one that carbon dioxide is necessary to the growth and development of the bacterial cell.

REFERENCES

D'Arsonval 1893 Remarques a propos des notes de MM. Sabrazès et Bazin Compt. rend. Soc. Biol., **5**, 914-16.

Buchner, Hans 1885 Beitrage zur Kenntnis der Neapeler Cholera Bacillus und einiger demselben nahestehender Spaltpilze. Arch. f. Hyg., **3**, 411-14.

Chapin, C. W. 1918 Carbon dioxide in the primary cultivation of the gonococcus. Jour. Inf. Dis., **23**, 342–43.

Cohen, M. B., and Markle, Louis 1916 A method which greatly facilitates the cultivation of the meningococcus. Jour. Amer. Med. Assoc., **67**, 1302-03.

COHEN, M.B., AND FLEMING, J. S. 1918 The diagnosis of epidemic meningitis and the control of its treatment by rapid bacteriologic and serologic methods. Jour. Inf. Dis., **23**, 337–41.

CORPER, H. J., GAUSS, H., AND REUSCH, O. B. 1921 Studies on the influence of carbon dioxide on resistance to tuberculosis. Amer. Rev. Tub., **5**, no. 7, 562–87; also Jour. Amer. Med. Assoc., **76**, 1216.

FRANKEL, CARL 1889 Die Einwirkung der Kohlensäure auf die Lebenstätigkeit der Mikroorganismen. Zeitschr. f. Hyg., **5**, 332-62.

GATES, L FREDERICK 1919 The effect of carbon dioxide in the cultivation of the meningococcus. Jour. Exp. Med., **29**, 321-38.

GODLEWSKY 1892 Bull. Intern. Acad. Sci. Cracowie, 408-57.

GODLEWSKY 1895 Bull. Intern. Acad. Sci. Cracowie, 178-92.

GODLEWSKY 1896 Centralbl. f. Bakt. Abt. II, **2**, 458-62.

GOWDA, R. N. 1924 Nitrification and the nitrifying organisms. Jour. Bacteriol., **9**, 251-71.

GROSSMANN, CARL AND MAYERHAUSEN 1881 Ueber das Leben der Bakterien in Gasen. Pflüger's Arch. f. Physiol., **15**, 248

HOCHSTETTER, M. 1887 Ueber Mikroorganismen im kunstlichen Selterswasser nebst einigen vergleichenden Untersuchungen uber ihr Verhalten im Berliner Leitungswasser und im destillierten Wasser. Arb. aus dem Kaiserl. Gesundheitsamte, **2**, 1-29.

HOFFMAN, W. 1906 Ueber den Einfluss hohen Kohlensauredrucks auf Bakterien im Wasser und in der Milch. Arch. f. Hyg., **57**, 379–400.

HUDDLESON, I. F. 1920 Studies in infectious abortion. Mich. Agr. College Exp. Sta. Tech. Bull., **49**, East Lansing.

HUNZIKER, O. F. 1924 Facts about carbonated butter. Jour. Dairy Sci., **7**, 484-96 (see page 491).

KOHMAN, ED. F. 1919 The so-called reduced oxygen tension for growing the meningococcus. Jour. Bacteriol., **4**, 571-84.

KOSER, S. A., AND SKINNER, W. W. 1922 Viability of the colon-typhoid group in carbonated waters and carbonated beverages. Jour. Bacteriol., **7**, 111-21.

KULP, W. L. 1926 The determination of viable *Lactobacillus acidophilus*. Science, **64**, no. 1656, 304-06.

LARSEN, W. P., HARTZELL, T. B., AND DIEHL, H. S. 1918 The effect of high pressures on bacteria. Jour. Inf. Dis., **22**, 271-79.

LEONE, C. 1886 Untersuchungen über die Mikroorganismen des Trinkwassers und ihr Verhalten in Kohlensauren Wässern. Arch. f. Hyg., **4**, 168-75.

LIESKE, RUDOLF 1911 Beiträge zur Kenntnis der Physiologie von Spirophyllum ferrugineum (Ellis). Jahrb. f. wiss. Bot., **49**, 91-127.

NOWAK, JULES 1908 Le bacille de Bang et sa biologie. Ann. de l'Inst. Past., **22**, 541-55.

NOVY, F. G., AND SOULE, M. H. 1925 Microbic respiration. Respiration of the tubercle bacillus. Jour. Inf. Dis., **36**, 168-232 (109 232).

PRESCOTT, S. C., AND PARKER, M. E. 1924 The effect of carbonation on bacteria in commercial ice cream. Cambridge, Mass. 16 pages.

PRUCHA, M. J., BRANNON, J. M., AND AMBROSE, A. S. 1922 Does carbon dioxide in carbonated milk and milk products destroy bacteria? Cir. 256, Ill. Agr Exp Sta . Urbana

PRUCHA, M. J., BRANNON, J. M., RUEHE, H A   1925   Carbonation of butter   Jour. Dairy Sci., **8**, 318–29

RETTGER, LEO F , WINSLOW, C -E A., AND SMITH, ARTHUR H   1922   Report of an investigation into the effect of freezing ice cream in an atmosphere of carbon dioxide.   National Assoc Ice Cream Manufacturers.

ROCKWELL, G E.   1921   The study of the gaseous requirements for the growth of various bacteria.   Jour Inf. Dis., **28**, 352–6.

ROCKWELL, GEORGE E.   1924   An improved method for anaerobic cultures   Jour. Inf. Dis , **35**, 580–86.

ROCKWELL, GEORGE E , AND HIGHBERGER, J. H.   1926   Carbon dioxide as a factor in the growth of the tubercle bacillus and of other acidfast organisms   Jour. Inf. Dis., **38**, 92–100.

SABRAZÈS. J., ET BAZIN, ED.   1893   L'acide carbonique a haute pression peut-il etre considere comme un antiseptique puissant?   Compt. rend. Piol. Ser , 9, **5**, 909 14.

SMITH, THEOBALD   1924   Some cultural characters of *Bacillus abortus* (Bang) with special reference to CO_2 requirements.   Jour Exp. Med , **40**, 219 32

SMITH, THEOBALD   1926   Variations in CO_2 requirements among bovine strains of *Bacillus abortus*   Jour Exp Med , **43**, no. 3, 317–25

STARKEY, ROBERT L   1925   Concerning the physiology of *Thiobacillus thiooxidans*, an autotrophic bacterium oxidizing sulphur under acid conditions.   Jour Bacteriol , **10**, 135–65

ST. JOHN, J. H.   1919   Oxygen tension in its relation to the meningococcus   Med. Rec , **95**, 184–86

SZPILMAN, JOS.   1880   Ueber das Verhalten der Milzbrand Bacillen in Gasen.   Zeitschr. physiol. Chem , **4**, 350.

TORREY, J. C , AND BUCKELL, , G. T.   1924   Cultural methods for the gonococcus.   Jour Inf. Dis , **31**, 125.

VINOGRADSKY, S.   1890   Ann. de l'Inst Past , **4**, 257.

VINOGRADSKY, S.   1899   Centrbl. f. Bakt , 2 abt., **5**, 329, 377, 429

WAKSMAN, S. A , AND STARKEY, R. L.   1922   On the growth and respiration of sulphur oxidizing bacteria.   Jour. Gen. Phys., **5**, 285–310.   See also STARKEY, R. L.   1925   Jour. Bacteriol., **9**, 161–95.

WHERRY, WM B., AND ERVIN, D. M.   1918   The necessity of carbon dioxide for the growth of *B. tuberculosis*.   Jour. Inf. Dis., **22**, 194–97.

WHERRY, WM. B., AND OLIVER, WADE W.   1916   Adaptation to certain tensions of oxygen as shown by gonococcus and other parasitic and saprophytic bacteria.   Jour. Inf. Dis., **19**, 288–98.

COLOR DIFFUSION IN ENDO AGAR

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Bacteriologists have long recognized the need for a reliable test to detect the presence of colon organisms and to differentiate between the various species of that group. In 1904 Endo published a test which has been recognized ever since as a valuable and a desirable one. It has been widely used, especially in this country, for studying members of the colon group of bacteria.

This medium contains an indicator in the form of decolorized basic fuchsin. When *Bact. coli* is grown on Endo agar it brings about a reaction which leads to a restoration of the color of the fuchsin in the colony while other bacteria have no such effect on decolorized fuchsin. This is an excellent and efficient method for detecting *Bact. coli* in contaminated materials, but it has certain disadvantages which have not, as yet, been entirely overcome.

The method for making this medium is described by Endo as follows:

Neutral 3 per cent agar broth	1000	cc.
Lactose C P.	10	grams
Alcoholic fuchsin	5 0	cc.
10 per cent sodium sulphite solution.	2 5	cc.
10 per cent sodium carbonate solution.	10 0	cc

Cook the agar broth well, filter, neutralize and then add 10 cc. of a 10 per cent solution of sodium carbonate to make it alkaline. Then add the lactose and fuchsin solution. This will make the medium red. Now add the sodium sulphite solution which will gradually decolorize the medium so that it will be entirely colorless when the agar is cold. After the medium has been put into tubes or flasks, it is sterilized for thirty minutes in the autoclave.

Precautions

1. The lactose must be chemically pure, for the commercial lactose contains ordinary cane sugar with which the typhoid bacillus will make acid and it will be very hard to distinguish it from the colon bacterium.

2. The sodium sulphite solution must be stored in a tightly stoppered flask or made up fresh.

3. The alcoholic fuchsin must be filtered beforehand.

4. The medium must be stored in the dark, otherwise by exposure to light the red color returns. When the medium is used, it is melted and poured into sterile plates and left standing uncovered in a dust free place until the plates harden. After they have hardened the plates will be colorless and transparent.

GROUPS OF COLON BACTERIA

There are four distinct classes of organisms belonging to the colon group which are classified according to their ability to ferment ordinary laboratory sugars:

    a. Those organisms which ferment glucose and lactose with the production of both acid and gas in each.

    b. Those which produce acid and gas in glucose, but which do not ferment lactose.

    c. Those which produce acid only in glucose and lactose.

    d. Those which produce acid only in glucose and do not ferment lactose.

The individual species within these groups ferment various other sugars, but for the purpose of this article these reactions are not essential.

Because of the varying ability of these organisms to ferment lactose, it is possible to detect members of the groups outlined above on Endo agar. Those which ferment lactose with the production of acid and gas are able to restore the color of the decolorized fuchsin, while those which produce acid only in lactose restore but a faint pink color, and the colonies of those organisms which do not ferment lactose remain colorless.

THEORIES FOR COLOR PHENOMENA IN ENDO AGAR

The reason for this color phenomenon has been explained in various ways by different workers, Endo (1904) explains it as follows:

The chemistry of the color change in the medium is caused by the following means: Fuchsin contains essential acid salts of rosanilin, $C_2H_{19}N_2HCl$. Rosanilin is a colorless, so-called leucko-base which with different acids, such as lactic acid, hydrochloric acid, etc. produces a red color. The acid compound of red rosanilin salts, is easily reduced through a reducing agent such as sodium sulphite. This, the colorless rosanilin combines with, through the production of acid by the colon bacillus, and the medium becomes a bright red.

Harding and Ostenberg (1912) commenting on Endo's explanation of the chemical change causing the restoration of color say that it offers a plausible explanation which seems to have been accepted, but it apparently was not based on experimental investigation. A summary of the explanation of the color phenomenon, by these authors, is, that the colorless solution obtained by adding fuchsin to sodium sulphite or sodium bisulphite or sodium dioxide in solution is used in chemical analysis for the quantitative determination of aldehydes. This solution is known as Schiff's aldehyde reagent. Acids do not give any color test with this reagent and, if present in sufficient quantity, prevent aldehydes from giving it. These authors quote Nietski as giving the following reaction between fuchsin, sodium sulphite and aldehydes: "Rosanilin and pararosanilin form with sulfurous acid and the alkali bisulfites colorless, easily decomposed compounds. By the action of aldehydes upon these bodies peculiar violet dyes are formed."

Robinson and Rettger (1916) have still another theory. They explain the reddening of the colon colonies by the presence in the medium of decomposition products of lactose, such as lactic acid and some acetic acid. These acids cause oxidation of the colorless leuco-base to the fuchsin color product. The decolorization, which takes place if the plate is allowed to stand long

enough, is due to such alkalis as ammonia and amines, which are formed from the peptone and the beef extract.

DeBord (1917) performed some experiments to test the effect of acid alone, combinations of acid and formaldehyde, and formaldehyde alone on decolorized basic fuchsins as well as on Endo agar. He thinks the reaction is an acid-aldehyde reaction and that the presence of an aldehyde is necessary to it.

MODIFICATIONS OF ENDO'S METHOD

This medium has become generally known as Endo's medium or Endo agar and has been in general use in bacteriological laboratories since its discovery. Efforts have been made from time to time to modify it, in order to correct some of the defects which lead to a lack of uniformity of results among different workers. Endo added 10 grams of sodium carbonate "to make the medium alkaline." This has been found to be too empirical a method of adjusting the reaction. Accordingly, careful titration of the amount of acid with phenolphthalein as an indicator and the use of NaOH to adjust the reaction to the desired alkalinity has been substituted. A still more accurate method is to determine the hydrogen ion concentration and adjust the reaction by this method.

In spite of a more carefully adjusted alkalinity, there is still some difficulty in securing a satisfactory medium. The chief trouble has probably been found with the color diffusion. Endo agar, prepared for use in the laboratory, develops a decided color on standing. Endo encountered this difficulty, and cautions that the medium must be kept in the dark as exposure to light causes a return of color. Later it was found that even slight amounts of acid caused the return of color in a sterile medium or might even prevent the complete decolorization of the agar. The importance of an alkaline reaction is thus emphasized.

Robinson and Rettger (1916) state that the final reaction and the use of *pure reagents, especially the lactose and sulphites* are the most important factors in securing a satisfactory Endo agar. They recommend the use of C.P. anhydrous sodium bisulphite

as a decolorizing agent, since poor results have been obtained with both crystalline and anhydrous sodium sulphite.

Levine (1921) has tried to solve the problem by substituting a dipotassium salt for the beef extract, which he believes simplifies the process and makes for a better differentiation between *Bact. coli* and *Bact. aerogenes*. He does not adjust the reaction of this medium, but states that *"The diffusion of color due to the production of acid is very rapid."*

Genung (1924) suggests that the restoration of color may be due to the pepton, having found two different brands of pepton resulted in marked differences in the color on sterile Endo agar plates incubated for twenty-four hours.

Harris (1925) finds that the hydrogen ion concentration and the brand of pepton, both have definite effects on the restoration of color in uninoculated plates.

IMPORTANCE OF A CLEAR CUT REACTION IN DETECTING THE KIND OF ORGANISMS PRESENT ON THIS MEDIUM

There is a marked difference in the degree of color produced by different species of the colon group of bacteria on this medium. *Bact. coli* colonies produce a deep, iridescent, fuchsin red, while the *Bact. aerogenes* colonies are a rose pink. *Bact. dysenteriae* and *Bact. para-typhosum* A have pale pink colonies, while *Bact. typhosum* and *Bact. typhosum* B colonies remain colorless. If the medium becomes highly colored, it diffuses into the growth so that typhoid colonies, for example, may become pink and resemble the colonies of dysentery or of *Bact. aerogenes*. It is important, if careful differentiation is desired, to have a medium which will remain colorless, except in the region of the colonies.

For several years the writer experienced considerable difficulty in securing a colorless Endo agar for class room work. Various brands of both crystalline and anhydrous sodium sulphite were tried and care was taken to have the reaction decidedly alkaline, but these precautions did not eliminate the difficulty. In connection with some laboratory experiments, Endo agar had been made up with two different brands of pepton. In testing the sterility of this agar by twenty-four hours incubation of the

plates, it was found that the plates made from the "Difco" brand of pepton, ordinarily used in this work, were a decided pink; while those made from Witte's pepton remained colorless. All the plates were sterile. This led to further tests and a series of experiments were undertaken to study the effect of different brands of pepton on the color diffusion.

MODIFICATIONS NECESSARY TO FACILITATE THESE TESTS

Several modifications have been suggested by different workers in the preparation of this medium. None of these materially affects the composition as outlined in the original formula, but they have primarily to do with facilitating processes of preparation. In choosing the formula for these experiments, it was thought best to use the method recommended by the Committee on Standard Methods of Water Analysis (1923) since that is, probably, the one most generally employed in public health laboratories.

From the beginning it was found necessary to alter this method somewhat, not only to facilitate the preparation, but to eliminate all possible chances of contamination. The method finally worked out and used in these experiments is given in some detail here, because it has frequently been charged that the method used in preparation accounts for the differences in results obtained by different workers. The medium used in these tests was as follows:

Beef extract. ..	5 grams
Pepton	10 grams
Distilled water .	1000 cc.
Agar........ ...	30 grams

The beef extract and pepton were dissolved in water and the agar added. The mixture was brought to a boil and autoclaved at fifteen pounds pressure for twenty minutes. At the same time the filters and flasks were sterilized. After removing from the autoclave the reaction was adjusted, 10 grams of lactose were added and the medium filtered into flasks. It was again autoclaved at ten pounds pressure for fifteen minutes.

When ready for use, to each 100 cc. of the agar was added 10 cc. of a 2.5 per cent solution of sodium sulphite in which 0.5 cc. of an alcoholic solution of basic fuchsin had been decolorized. It was immediately poured into plates and allowed to harden before replacing the covers.

The method which was used for titrating the reaction was the colorimetric one recommended by Jordan (1924). To 4 cc. of heated distilled water was added 1 cc. of the agar and five drops of Brom Thymol Blue as indicator. The medium was carefully adjusted to the desired hydrogen ion concentration and the reaction rechecked. This method was found to be most satisfactory as the highly diluted agar reduces the error caused by the masking of the color of the indicator in a less dilute medium. It also checked with the other methods in accuracy.

TESTS WITH DIFFERENT BRANDS OF PEPTON

The first factor which was tested was the pepton factor. Six different brands of pepton on the market for culture media work were secured. They were Witte's, Armour's, Difco, Fairchild's, Parke-Davis' and Merck's. The latter has been discontinued since these tests were begun. Endo agar was prepared using these peptons and plates were poured. They were allowed to harden and then divided into three groups, one set was inoculated with *Bact. coli* and one with *Bact. aerogenes* while the third was not inoculated. Since exposure to light has always been thought to have an effect on the production of color, the experiment included this factor. Uninoculated plates were left exposed to the daylight, while others were exposed to the light of an ordinary electric light bulb. The remainder were kept in the dark. It was found to be inadvisable to inoculate the daylight plates as the colonies never grew well. Table 1 shows the results of this experiment. The work was carefully rechecked many times at varying intervals and the results were found to be uniform.

There is the least return of color in the plates containing Witte's pepton agar while Armour and Merck pepton agars show the greatest amount of color. The amount of color at the end

of twenty-four hours incubation was comparatively small even in those plates which were exposed to light, when plates made from the same pepton were compared. After being incubated for an additional twenty-four hours, there was still practically no difference in the relative amounts of color in plates of agar made from the same brand of pepton, whether the plates were incubated in the dark, in artificial light or exposed to

TABLE 1

The effect of peptones and light on restoration of color in endo agar

PEPTONE	DAYLIGHT EXPOSURE	DARK INCUBATOR		LIGHT INCUBATOR	
		Inoculated	Not inoculated	Inoculated	Not inoculated
After twenty-four hours					
Witte..................	−	−	−	−	−
Parke-Davis........................	+	+	+	−	−
Fairchild..........	+	+	+	+ +	+ +
Difco....	+	+	+	+	+
Merck....	+ +	+ +	+ +	+ +	+
Armour..................	+ +	+ +	+ +	+ +	+ +
After forty-eight hours					
Witte.........	+	+ +	−	+ + +	+
Parke-Davis	+ + + +	+ + +	+	+ + + +	+
Fairchild	+ +	+ + +	+ +	+ + +	+ +
Difco..........	+ + +	+ + +	+ +	+ + + +	+
Merck..................	+ + +	+ + + +	+ +	+ + + +	+
Armour...........................	+ +	+ + + +	+ +	+ + + +	+

− no color visible; + faint pink tinge; + + distinct pink tinge; + + + whole plate rose pink; + + + + whole plate deep rose red.

daylight. The return of color after the first twenty-four hours incubation was more marked and took place more rapidly than during the first period.

The different brands of pepton were tested for their pepton content by a chemist. The results of these tests are shown in table 2. This table shows their comparative solubility and the color of the solutions. According to the biuret test these peptones vary considerably in their actual pepton content. An

interesting fact may be pointed out in connection with the Parke Davis product. This firm discontinued their old brand of pepton and replaced it with a new brand which they labelled, "Bacteriologic Pepton." This new product gave a negative biuret test while the discontinued product was fairly high in

TABLE 2

Tests for Pepton Content

Solutions: 1 gram in 100 cc. of distilled water

BRAND	pH	SOLUTION	COLOR OF SOLU-TION	BIURET TEST*	REMARKS
Witte	7 0	Heavy suspension	Colorless	++	
Parke-Davis	6 4	Slight suspension	Very slight coloration	++	
†Parke-Davis	5 7	Slight suspension	Very faint yellow	−	Not rose pink of true peptone. Purple with strong pink tinge.
Fairchild	5 4	Not perfect	Deep yellow	−	A large excess of 40 per cent NaOH brings out the biuret pink
Difco	7 0	Perfect	Faint yellow	+	
Armour	6 2	Perfect	Faint yelow	+	
Merck	4 0	Fine suspension	Orange	−	With excess of 40 per cent NaOH color is purpose-pink

+ positive test; ++ strongly positive test; − negative test.
* One cubic centimeter of 1 per cent NaOH + 3 drops of 2 per cent $CuSO_4$.
† New product of the Parke-Davis Company called "Bacteriologic Pepton."

pepton content. Another fact which this test brought out was, that Witte's pepton, which seems to inhibit the return of color to a greater extent than other brands, contains a heavy insoluble precipitate in water which does not dissolve when the mixture is heated.

Since the reaction of the medium appears to influence the amount of color production, the agar was adjusted to various

reactions from pH 7.0 to 7.8. It was found that the growth of the organisms was inhibited when the range was above pH 7.6 and that a pH of 7.0 was not alkaline enough, since the plates were all highly colored. A careful test was made of the comparative effects of pH 7.2 and 7.4 on the color. These results are shown in table 3. There is relatively little difference in the

TABLE 3

The relation of reaction of medium to restoration of color

PEPTONE	pH 7 2				pH 7 4			
	Daylight	Dark	*Bact coli*	*Bact aerogenes*	Daylight	Dark	*Bact coli*	*Bact aerogenes*
After twenty-four hours								
Witte	−	−	−	−	−	−	−	−
Parke-Davis . . .	−	−	+	+	+	−	+	−
Fairchild	−	−	+ +	+ + +	+	+	+	+
Difco	+	+	+ +	+ +	+	+	+	+
Merck	+ + +	+	+	+ +	+ +	+	+ +	+ +
Armour.	+	+	+ +	+ + +	+ +	+ +	+ +	+ +
After forty-eight hours								
Witte	−	−	+ +	+ +	−	−	+ +	+
Parke-Davis	−	+	+ + +	+ + + +	−	−	+ +	+
Fairchild . .	+	+	+ +	+ + + +	+	+	+ + +	+ +
Difco	+	+	+ + +	+ + + +	+	+	+ + +	+ +
Merck	+ + +	+	+ + +	+ + +	+	+	+ + + +	+ +
Armour	+	+	+ + +	+ + + +	+	+	+ + +	+ + +

— no color visible; + faint pink tinge; + + distinct pink tinge; + + + whole plate rose pink; + + + + whole plate deep rose red.

amount of color production in the different pepton agars between the two reactions. A pepton which does not inhibit color production has about the same depth of color in both reactions. After forty-eight hours incubation, the color is more marked in the pH 7.2 plates. Since there was this slight difference, the reaction pH 7.4 has been used in all subsequent work.

FUCHSIN AS A FACTOR IN COLOR DIFFUSION

During these tests it became evident that the fuchsin played an important part in the return of color. Accordingly a careful study was made of this factor. Endo (1904) stated that 5 cc. of basic fuchsin was to be added to each liter of agar. He gave no directions as to the strength of the solution, only stipulating that it must be filtered. Later authors recommended a saturated alcoholic solution of basic fuchsin. A saturated alcoholic solution of basic fuchsin is usually made by adding one part of the dry fuchsin to ten parts of 95 per cent alcohol. This mixture is allowed to stand twenty-four hours then the supernatant fluid is filtered off and used in the Endo agar. The common practice is to add 5 cc. of this fuchsin solution to each liter of agar, together with 10 cc. of a 2.5 per cent solution of sodium sulphite to decolorize the fuchsin.

DIFFICULTIES ENCOUNTERED WITH AMERICAN FUCHSINS

When American brands of fuchsin came into the laboratory, much difficulty was encountered in decolorizing the agar by this method. It was found to be more satisfactory to add the fuchsin to the sulphite solution before adding it to the agar. This method resulted in the discovery that different makes of American fuchsin did not decolorize in the sodium sulphite solution in the previously mentioned proportions. The fuchsin not only failed to become decolorized, but formed a thick, flocculent precipitate in many cases. Careful tests of a large number of fuchsins were made, in order to discover their ability to become decolorized in the sodium sulphite solution. The results of these tests have already been published (Genung, 1926a) so that it is necessary to give only a brief summary here.

It was found that different lots of fuchsin, even from the same firm, vary in their solubility in alcohol (Conn, 1923) and that further dilution is often necessary before they will be decolorized by the sodium sulphite solution. A scheme was devised whereby each new lot of fuchsin could be tested for this property. A series of dilutions of the saturated, alcoholic solution of the

fuchsin were made and each was tested in a sodium sulphite solution of the same strength and in the same proportions used in Endo agar. The lowest dilution which was decolorized by the sodium sulphite solution was used in the agar. This was further tested by inoculating plates made with this solution of the fuchsin with known organisms. *Bact. coli* and *Bact. aerogenes* were used and if these organisms produced characteristic reactions on the agar, the dilution was considered satisfactory. It was also found by these tests that some fuchsins were never satisfactory. They either would produce too much color in low dilutions or too little color in higher dilutions or sometimes they were not sufficiently decolorized by the sodium sulphite solution, even in high dilutions, to make it advisable to use them for this work.

When carefully tested and properly diluted fuchsin was used there was the same general variation in color return in the plates of agar made from the different peptones. These variations remained uniform throughout the experiments. If a pepton did not inhibit color production in the agar, its effect was only accentuated when too strong a solution of fuchsin was used; while a pepton which inhibited the color production had a deeper color in the stronger fuchsin solutions. It would seem that the fuchsin factor is an important one, but at the same time, one capable of solution.

THE EFFECT OF LIGHT ON COLOR PRODUCTION

As was noted in a previous paragraph, experiments were carried on to test the effect of exposure to light on the return of color in Endo agar. Various tests of this factor were made. In each batch of plates a certain percentage were exposed to both daylight and artificial light. Tubes and flasks of the agar were also exposed to light and compared with duplicates kept in the dark.

The results of these tests with the plates are recorded in table 1. They seem to indicate that light as a factor in the return of color in Endo agar is fairly negligible. When exposed to artificial light or daylight, the plates developed very little more color than

those kept for the same length of time in the dark. The different peptones showed characteristic reactions in either case. Those where the color diffusion was marked developed the same degree of color in the dark as in the light. Those peptones which inhibited color production developed but little color in the light. After twenty-four to thirty-six hours incubation, the return of color was more rapid in the light, but they all ultimately became a deep, dark red at the end of forty-eight hours. Artificial light had less effect on the rapidity of color diffusion than daylight. This fact is rather interesting, since the workers in one laboratory, known to the writer felt they could not use Endo agar because their incubator was heated with electric light bulbs. Agar treated in the same way in larger masses, such as tubes and flasks, showed even less effect of light. The return of color was more gradual than in the plates, but it seemed to be uniform in either condition.

ORGANISMS USED TO TEST THE EFFICIENCY OF THE MEDIUM

In all these experiments, two organisms were used to test the effects of these different factors on the agar. *Bact. coli* and *Bact. aerogenes* were chosen because they produce definite, but different reactions on Endo agar. *Bact. coli* gives a characteristic, iridescent, fuchsin red colony; while *Bact. aerogenes* produces a rose pink color in the growth. Before completing the test of any one factor or combination of factors, inoculations were made with other organisms to compare their reactions with those ordinarily used. For these further tests *Bact. typhosum, Bact. paratyphosum* A, and *Bact. paratyphosum* B were used.

The results of the tests with these organisms showed that where the medium developed a deep red color, it would often diffuse into the colony and cause a normally white colony to become a rose pink.

MODIFICATIONS OF ENDO'S MEDIUM SUGGESTED BY DIFFERENT EXPERIMENTERS

There have been several attempts to alter the composition of Endo's Medium in order to improve it and to obtain more uni-

form results.   Robinson and Rettger (1916) found that sodium bi-sulphite used as a decolorizing agent, gave more satisfactory results than sodium sulphite and they recommended its adoption. Levine (1921) finds that the substitution of dipotassium phosphate in place of the beef extract gives better results, especially in the differentiation between certain species of the colon group of bacteria.

Some workers use 1.5 to 2 per cent agar instead of 3 per cent agar.   Levine advocates 2 per cent agar in his method.   Certain manufacturers are putting a dehydrated Endo agar on the market, the exact ingredients of which are not always known to the laboratory worker.   Since all these changes and modifications may enter into the problem of color production, they were studied and compared with the standard method.

In working with the method suggested by Robinson and Rettger (1916), it was found that when sodium bisulphite-fuchsin solution was added to the agar and the plates inoculated, no growth appeared.   The agar was apparently too acid for bacteria to grow since sodium bisulphite is highly acid.   Tests were made to ascertain how best to overcome this difficulty and it was found that by adjusting the reaction of the agar to pH 10.0 before adding the sodium bisulphite solution, the final reaction would be about pH 6.8.   Plates were made from this agar but the growth was very scanty or, in some cases, no growth at all appeared. Several failures to use this method successfully resulted in its, being discarded for these experiments.

Levine's method was carefully tested.   He states that it is not necessary to adjust the reaction, but in these tests plates were made from unadjusted medium and from the medium adjusted to pH 7.2.   The reaction of the agar made from the different peptones varied considerably, ranging from pH 6.4 to 6.8.   When plates of the Endo agar, containing these peptones and made according to Levine's formula, were inoculated with *Bact. coli* and *Bact. aerogenes*, the results were interesting.   In none of the plates did the *Bact. coli* colonies show the characteristic fuchsin red, on the contrary all the colonies were a deep rose red; those of *Bact. coli* being a trifle deeper color than those of *Bact. aero-*

genes. In the plates adjusted to pH 7.2, the colonies were a fainter pink or, in some cases, there was no color at all. There was no return of color in the agar in any of the plates at the end of twenty-four hours incubation. This did not seem to be a good medium for differentiating between *Bact. coli* and *Bact. aerogenes.*

The use of dehydrated Endo agar and of the standard Endo agar containing only 1.5 per cent agar were tried but the color in both cases was very marked. Neither method seemed to offer a solution to the problem.

In a previous article (Genung, 1926b), the writer has attempted to show that the hydrogen ion concentration of the pepton has a direct effect on the return of color in the fuchsin-sulphite solution. The different brands of peptones, as will be noted in table 2, differ in their acidity, ranging from a fairly high acidity in the case of Merck's to neutrality in Difco's and Witte's peptones. When the decolorized fuchsin was added to these pepton solutions, the color returned immediately in those which had an acid reaction and did not return at all in the neutral solutions. After all the solutions of pepton were adjusted to pH 7.0, no color returned upon the addition of the fuchsin-sulphite solution. The addition of a very minute amount of a weak acid solution and a small quantity of formalin restored the fuchsin color completely. This bears out the theory suggested by DeBord (1917) that the return of color in the colonies of the colon group on Endo agar is an acid-aldehyde reaction and not due to the presence of lactic acid alone.

Since the presence of very small amounts of acid and aldehyde affect the return of color to a marked extent, and since the peptones vary considerably in their actual pepton content, it is interesting to speculate as to their true composition and to question whether the presence of simple protein and carbohydrate compounds may not be, to some extent, responsible for the return of color in Endo agar. It is possible that there may be some significance in the fact that those brands of peptones which give positive biuret tests are the most nearly neutral in their reaction and have the least diffusion of color in the agar, while those which give a negative pepton test are highly acid and the color production is rapid and profuse.

SUMMARY

One of the disadvantages of Endo agar as a differential medium for use with the colon-typhoid group of bacteria is the return of the fuchsin color in the agar of both inoculated and uninoculated plates. This color may spread into the colony and hamper identification.

These experiments were conducted to find out, if possible, what factor or combination of factors caused the return of color and what could be done to prevent it. The formula recommended by the Committee on American Standard Methods of Water Analysis (1923) was used for preparing the agar for these tests. Certain modifications recommended by different workers were also tried, but they seemed to have no advantage over the Standard Method for eliminating color diffusion.

The effect of different brands of pepton, of different kinds and dilutions of basic fuchsin and of sodium sulphite and sodium bisulphite as decolorizing agents were tested. The reaction of the agar and the effect of exposure to light were also considered.

It was found that the two most important factors in color production seemed to be the kind of pepton and the strength of the alcoholic solution of the basic fuchsin, used as an indicator. Peptones giving a positive biuret test seemed to inhibit the color production more definitely, than those which gave a negative test for pepton content.

The American fuchsins differ in their solubility in alcohol so that a fuchsin carefully tested for the proper dilution is less apt to cause return of color in the agar. Sodium sulphite is far more satisfactory as a decolorizing agent than sodium bisulphite.

The reaction of the medium is also important and should be adjusted to at least pH 7.4 as acidity causes a rapid return of the color. Exposure to light is a fairly negligible factor as compared with the others.

CONCLUSIONS

This study of color production in Endo agar has not entirely solved the problem, but it is clear that it offers certain possibilities whereby the condition may be improved.

Endo agar is an excellent medium for detecting the presence of and differentiating between different members of the colon group of bacteria. In order to secure clear cut results, it is desirable to have as little restoration of color in the agar as possible, during the incubation period. Several factors seem to affect this return of color, many of which may be modified or controlled.

The peptones used in preparing the agar may have an effect on the production of color and this seems to be in direct correlation with their actual pepton content.

The reaction of the medium is important in checking color diffusion. A decidedly alkalin reaction, ranging from pH 7.2 to 7.6, does not affect the growth of the bacteria and, at the same time, prevents a too rapid return of color in the plates.

The strength of the fuchsin solution affects the production of color. Since the different lots of American fuchsins, appearing on the market, differ in their solubility in alcohol, it is more satisfactory to use a fuchsin which has been carefully tested for its ability to be decolorized in the sodium sulphite solution.

The effect of light on the return of color during the first twenty-four hours is fairly negligible, and, if other factors are carefully controlled, this factor need not be considered. By adding the fuchsin-sulphite solution immediately preceding the pouring of the plates any problem of return of color during storage may be eliminated.

Various modifications of Endo agar have been suggested, but none of them seem to have any marked advantage over the standard method. On the contrary, some of them do not appear to be as satisfactory as the standard method.

Endo agar, made up with a high grade of pepton and a carefully tested solution of fuchsin, together with a carefully adjusted alkalinity, is an excellent differential medium for studying members of the colon-typhoid group of bacteria.

REFERENCES

CHAMOT, E. M., AND SHERWOOD, C. M. 1917 Culture media employed in the bacteriological examination of water. Jour. Amer. Chem. Soc., **39**, 1755-1766.

CONN, H. J. 1922a American biological stains compared with those of Grübler. Science, **55**, 284–285.

CONN, H. J. 1922b Preliminary report on American biological stains. Science, **56**, 156–160.

CONN, H. J. 1923a The preparation of staining solutions. Science, **57**, 15–16

CONN, H. J. 1923b Dye solubility in relation to staining solutions. Science, **57**, 638–639.

CONN, H. J. 1924 A report on basic fuchsin. Science, **60**, 387–388.

CONN, H. J. 1925 Biological Stains. The Commission, Geneva, N. Y.

DeBORD, G. G. 1917 The fuchsin-aldehyde reaction on the Endo medium. Jour. Bact; **2**, 309–314.

ENDO, S. 1904 Uber ein Verfahren zum Nachweis der Typhusbacillen. Cent fur. Bakt. I. abt., **35**, 109.

GENUNG, E. F. 1924 Endo agar as affected by peptone. Science, **59**, 282.

GENUNG, E. F. 1926a Basic fuchsin as an indicator in Endo's medium. Stain Tech., **1**, 41.

GENUNG, E. F. 1926b A consideration of French's test of basic fuchsin for Endo's medium. Stain Tech., **1**, 135.

GREY, E. C. 1913 The production of acetaldehyde during the fermentation of B. coli communis. Biochem. Jour., **7**, 359–363.

HARDING, E. R., AND OSTENBERG, Z. 1914 Studies on Endo's medium with observations on the differentiation of bacilli of the paratyphoid group. Jour. Infect. Dis., **11**, 109.

HARRIS, N. MacL. 1925 The preparation of Endo Medium. The Military Surgeon, **57**, 280.

JORDAN, E. O. 1924 General Bacteriology. Saunders Company.

KASTLE, J. H., AND ELVOVE, E. 1909 On the use of anhydrous sodium sulphite in the preparation of Endo's medium. Jour. Infect. Dis., **6**, 619.

LEVINE, MAX, 1921 Bacteria fermenting lactose and their significance. Iowa St. Col. Eng. Bul. **62**.

ROBINSON, H. C., AND RETTGER, L. F. 1916 Studies in the use of brilliant green and a modified Endo's medium in the isolation of *Bacillus typhosus* from feces. Jour. Med. Res., N. S., **29**, 363.

Standard Methods of Water Analysis. 1923 Amer. Pub. Health Assoc., New York.

AN APPARATUS FOR MOTION PHOTOMICROGRAPHY OF THE GROWTH OF BACTERIA

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The use of the motion picture camera with the microscope offers advantages in bacteriological research which cannot be entirely fulfilled by still photography or by the camera lucida. Chief among these advantages is the opportunity for continuous record of every phase of development at a minimum expenditure of time on the part of the worker.

Biologists, in the past, have devised various forms of motion picture apparatus to gain this advantage of a continuous photographic record of the movement of leucocytes, the mitosis and growth of cells in tissue culture, and the circulation of corpuscles in the capillaries. Undoubtedly, the published descriptions of mechanisms used and the films obtained do not include all the attempts which have been made to apply motion photomicrography to the solution of biological problems. References to some of these accounts are cited in the bibliography. The contributions to anatomical and physiological knowledge contained in these reports give evidence of the value of the method. These reports, however, did not influence the development of the apparatus to be described in this paper. Recent improvements in the motion picture camera and film permitted us to use "stock" material in making an apparatus which is relatively simple in construction and easy to operate in a bacteriological or biological laboratory.

Hitherto, the use of motion pictures for such study has been limited by the considerable expense involved in the purchase of standard (35 mm.) motion picture equipment and by the difficulty and inconvenience involved in the processing of 35-mm. film.

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Both of these difficulties have been minimized by the advent of the 16-mm. film and the equipment for its use.

A model A Ciné Kodak was used in these experiments. The camera was recommended by the Eastman Kodak Company as being much better suited to the needs of photomicrography than the Model B which is intended only for general photography. The taking lens of the Model A had to be removed, otherwise no changes in the stock camera were required.

The auxiliary equipment for successful motion photomicrography of living organisms may be classified under two heads: (1) A viewing device which will permit of accurate focusing and adjustment of the object within the confines of the small picture frame of the Ciné Kodak film. Both of these factors should be under the control of the operator while the picture is being made. (2) An automatic exposure device which will take pictures at intervals commensurate with the activity of the organism. Such a timing device should work with as little vibration as possible and should be capable of large variation in the frequency of exposure.

THE VIEW FINDER

Either the demonstration eyepiece or the binocular microscope seemed to offer a possible solution to the need of inspecting and focusing the object while pictures are being taken. Both of these were tried with indifferent success. The principal difficulty with either was that excessive magnification was unavoidable. In order that the focus of the virtual image seen in one eyepiece should coincide with the focus of the real image projected on the film by the other eyepiece, it was necessary to place the camera at a distance of approximately 25 cm. from the microscope. When this was done, it was found that the size of the field included by the frame of the Ciné Kodak film was disappointingly small, less than one-hundredth of the area seen in the other ocular. The resulting magnification was much greater than necessary for most subjects.

After trying one or two other devices for viewing the field, it was decided to construct a beam-splitting attachment to meet
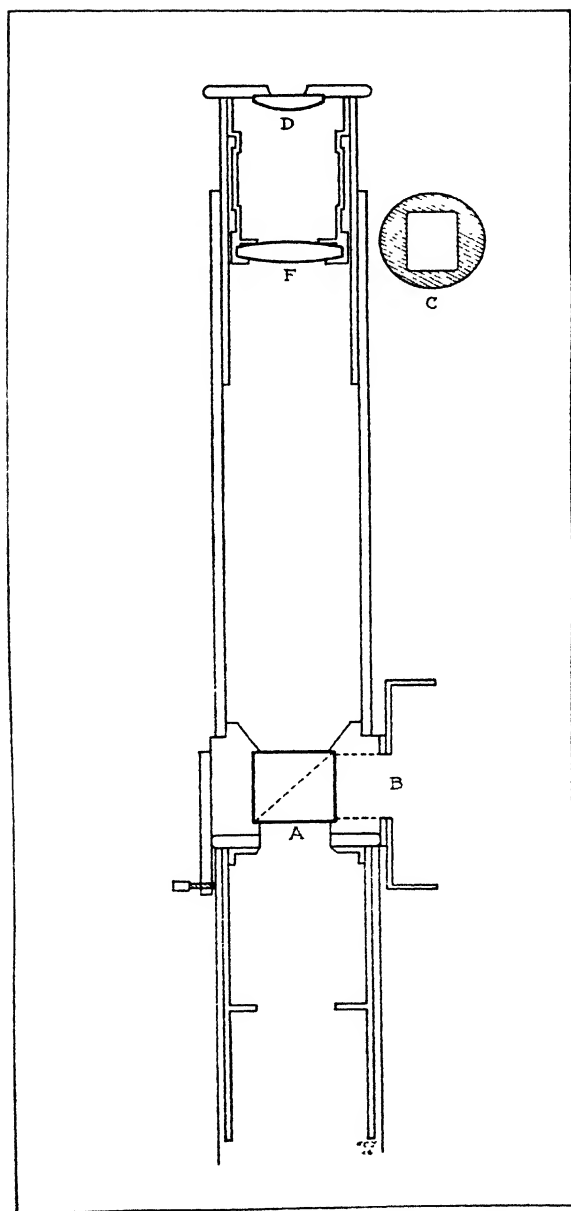
FIG. 1. LONGITUDINAL SECTION OF VIEW-FINDER

A, glass cube, right-angle prisms, with reflecting surface, *B*, aperture with flanged joint to fit camera tube, *C*, plane of image formed in view-finder, with mask corresponding to film slit, *D*, eye-lens, *E*, tube with fixed diaphragm to prevent internal reflection, *F*, field lens.

our special requirements. The microscope adapter illustrated in figure 1 was the result. The essential part of this device is the glass cube A. It is made up of two right angle prisms. The face of one was silvered by cathode sputtering and the two subsequently cemented together. The silvered face transmits about 10 per cent of the incident light, reflects about 85 per cent, and absorbs about 5 per cent. The reflected portion of the light passes out of the aperture B toward the film. The transmitted portion forms an image in the plane C. In this plane an opaque mask of the same size as the Ciné Kodak frame designates the limits of the field. The film and visual frame are equidistant from the center of the glass cube so that when the focus is sharp at C it is also sharp on the film. A field lens, F, of 4 cm. focal length collects the light from the visual image and an eye lens D magnifies this image about six times. A tube E with a fixed diaphragm to prevent reflections from the inside of the microscope barrel fits into the end of the microscope in place of an ocular.

SUB-NORMAL TAKING SPEEDS

For reactions such as the growth of bacteria or fungi, it is of value to speed up the rate of change as seen on the screen. If several hours change can be condensed to a viewing time of several minutes, there results a great saving of film and an added value as scientific record or material of educational interest. The usual type of stop motion apparatus for motion picture work is some form of clock controlled intermittent mechanism which will make exposures at predetermined intervals. The adaptation of such apparatus to the Ciné Kodak presented some difficulties of mechanical construction and it was decided to try a continuous movement driven by an electric motor through worm reduction gears. A small $\frac{1}{200}$ h.p. universal motor was used which has a no-load speed of about 2000 r.p.m. on 110 volts. It was found that by decreasing the voltage the speed of the motor under no load could be decreased to about 500 r.p.m. without too great loss of driving force. For pictures of the development of bacteria a speed of about 1 to 2 pictures per minute was judged to be

desirable. Since the Ciné Kodak is geared to make 8 pictures for a single revolution of the crank shaft reduction gears were required which would give a ratio of 1:4000. Two worm and spur gears were used in series and mounted together in a housing. Each of the gears had a ratio of 1:72 and 1:92 so that with this arrangement it is possible to get exposure speeds ranging from $\frac{1}{2}$ per minute to 10 per minute by the use of rheostat control of the voltage.

When the camera is running at these extremely slow speeds, the actual time of an exposure is very much greater than at the normal exposure rate of 16 per second. With pictures made at the rate of 1 per minute, the exposure time is one-half minute and the light intensity may be reduced to a very low level. This is a decided advantage when working with organisms which might be injured by the intensity of the light required for short exposure time.

THE MICROSCOPE AND ITS OPTICAL EQUIPMENT

A Bausch & Lomb microscope equipped with an aplanatic substage condenser, mechanical stage, and apochromatic objectives was used. It is believed, however, that an Abbé type condenser and achromatic objectives would have served practically as well.

No oculars were used because none was necessary to get the required magnification and because the quality of the image at the image distance used was better without an ocular. It would have been possible, no doubt, to have obtained slightly better performance from the optical system had we made use of an achromatic negative lens in place of an ocular to preserve the tube length correction of the objectives.

It is extremely desirable in motion picture work with the microscope to keep the magnification at the lowest possible value. The motion picture frame is a very small area compared to the 5- by 7- inch or 8- by-10 inch-plates with which photomicrographers are accustomed to work. The greater the portion of the object slide included in the photograph, the more reliable the information which can be obtained from the picture. In still photography,

magnifications of 2000 × or more are common. Such a high value is not particularly undesirable on a 5- by 7-inch plate, but if a magnification of this order were to be used on the 16 millimeter motion picture, one organism 4 or 5 microns in length would go far toward filling the field. Consequently one such organism would about fill the motion picture screen when the film was projected. Such a picture could well be said to have "empty magnification."

Because of the special importance of the relation between magnification and resolving power in motion picture work, it will not be out of place to speak briefly of the fundamental principles which underlie the requirements of photomicrography. At a given tube length the magnification of an objective is fixed by its focal length. To this initial magnification we can add either by the use of an ocular or by projecting the image a greater distance. This added magnification we shall refer to as "secondary magnification." For a given numerical aperture, an objective is capable of distinguishing a certain fineness of detail irrespective of magnification. It is desirable that the sensitive photographic material shall be able to distinguish all the detail which the objective resolves.

Because of the granular structure of the image, a photographic material is capable of recording detail only if the dark and light areas which make up this detail are separated by a finite amount. The ability of an emulsion to record detail is spoken of as its resolving power and this usage is analogous to that of the same term applied to microscope objectives. Ciné Kodak film has a resolving power of about 50 lines per millimeter.

Returning now to the question of secondary magnification. If the initial magnification of the objective does not stretch out the detail so that the light and dark spaces of the finest structure which it can resolve are at least one-fiftieth of a millimeter apart, some secondary magnification is desirable. For example, a 2-mm. oil immersion objective of 1.3 N.A. has a resolving power of about 5500 lines per millimeter for a wave-length of 480μ. In order that the film may record this detail the magnification should be about 110 ×. For a tube length of 160 mm. the initial

magnification of this objective is $90\times$.   The required secondary magnification is $110/90 = 1.22$.   Upon computing the value of required secondary magnification for all the objectives which it was intended to use, it was decided that no ocular would be necessary and that the camera could be placed as close to the microscope as was convenient mechanically.

With the view finding device previously described the secondary magnification is $1.7\times$ which gives an ample factor of safety in the matter of resolving power and does not result in the loss of more object slide area than is necessary.

ADJUSTMENT OF APPARATUS

A camera stand adjustable in a vertical direction was devised for this work.   It consists of a base plate from which extend three cylindrical tubes.   Three rods supporting a top plate slide up and down with these tubes.   The movement is actuated by a rack and pinion acting on one of the rods and a set screw holds the camera in position after the vertical adjustment has been made.

The camera and motor drive unit are mounted on the stand. The camera lens is removed and into its place is screwed a connecting collar to exclude extraneous light.

The microscope is now turned so that it can be pushed up to the camera without making contact with either it or the stand.

The viewing device is placed in the microscope and the object slide is approximately focused.   The camera stand is racked up or down until the aperture in the viewing device and the connecting tube of the camera are at the same height.   The microscope is pushed gently up to the camera until the collar on the viewing device overlaps the tube on the camera.   The collar and the tube make a sliding fit so that the relative positions of film frame and viewing frame are accurately fixed.   The entire assembly is shown in plates 1 and 2.   It is desirable when the exposures are to be made more rapidly than 2 or 3 per minute to remove a slip ring from the inside of the collar so that the camera and microscope will be entirely separated.   This slip ring which is made to fit tightly in the collar on the viewing device can be

allowed to remain hanging on the connecting barrel. This procedure will prevent the transmission of camera vibration to the microscope.

It is sometimes essential that the camera stand and the microscope shall be supported by different tables placed close together but not touching each other. If work is to be done during the day time when the building is subject to vibration from passing traffic or from heavy machinery, it is necessary to support both tables upon shock absorbing mountings. The whole apparatus may be set on a platform which rests on inflated tubes or tennis balls. Four Spalding tennis balls of the solid variety at each corner of the platform are very effective in reducing vibration. At each corner, three of the balls should be arranged in the form of a triangle and kept from rolling apart by means of wooden frames. The fourth ball should be placed on top of the three to form a pyramid. The effect of vibration is more serious when working at high magnification and with preparations having free liquid surfaces.

Motion pictures may be injured by vibration in three distinct ways, and it is well to recognize the cause of the trouble before attempting to apply a remedy. Vibration of high frequency transmitted to the apparatus results in poor definition because of the trembling of the image during exposure. This effect can almost always be recognized by careful inspection of the visual frame of the viewing device. Occasionally even though vibration is not of the nature which causes perceptible trembling of the image, it may result in poor definition by jarring the objective out of focus if the apparatus is kept running for a long time. This effect is particularly troublesome if the fine focusing adjustment of the microscope works loosely. The obvious remedy is to tighten the adjustment or to provide some means of holding the tube firmly in position. The third difficulty is due to cranking vibration. The projected picture may be of good definition but appears unsteady on the screen. The cause, if it is not projection difficulty, may be attributable to unsteadiness of the camera on its support, careless cranking on the part of the operator, or poor alignment of mechanical parts if the motor drive is used.

EXPOSURE TRIALS

The problem of getting correct exposure for various conditions is a matter which requires a great deal of attention. Not only is the exposure dependent upon the equality and intensity of the light source, the magnification, the numerical aperture of the objective, and the cranking speed, but it is affected very markedly by the opacity and color of the specimen.

Sending the film to the processing laboratory involves considerable delay and loss of material if the exposure was greatly in error. It is much better to determine an approximately correct exposure by means of short length test strips, exposed in the camera and developed to a negative by the operator.

To this end, the camera is loaded as usual and the four feet of protecting paper leader which is wrapped around the outside of Ciné Kodak film is cranked off. The operator then proceeds to change whatever conditions of exposure are under his control and to select the best results.

Most of the variables by which the exposure is ordinarily regulated, are fixed in motion photomicrography by the conditions peculiar to the individual subject. The cranking speed is determined by the speed of movement or growth of the organism, a factor over which we have very little control. Other conditions of exposure are fixed with equal rigidity by the demands of resolving power and magnification. The operator has only one variable under his complete control, that is, the intensity of his light source. He can exercise an approximate control over the intensity by the use of different light sources and by the variation of the voltage which he uses with these sources. The most accurate method of controlling the intensity, although not the most efficient, is the absorption of light by means of filters. Two classes of filters are available for this purpose, the selective or colored filters and the non-selective or neutral gray densities. The use of a blue filter (No. 49B) is recommended for two reasons, first because of the better performance of the objective with that predominant wave-length of fairly homogeneous light and second because of the closer relation between the visual and photographic

focus. It may not be possible, however, to get exactly the correct amount of absorption using only colored filters and in this case neutral gray densities may be used to supplement the blue filter.

A series of exposure trials may be made up as follows:

1. A few pictures are exposed at the speed to be used, with no filter.

2. A single blue filter (No. 49B) is interposed between the source and the reflecting mirror.

3. A second blue filter (No. 49B) or various neutral densities are added.

After the exposure series is complete, the camera may be unscrewed from the stand and taken into the darkroom. The test strip is cut from the roll and the remainder of the roll is wrapped in black paper to be kept for future test strips.

The exposed film should be developed for five minutes at 68°F. in a solution of Elon-Hydroquinone tank developer such as can be obtained at any photographic supply house. The film can be handled easily in a one liter beaker. After the film has been fixed, an inspection will determine which has been the best exposure. In order to yield a good positive when the film is processed, the negative should show the required detail when held over a piece of white paper illuminated by bright sunlight. Some experience is necessary before it is possible to select the best exposure by an examination of the negative. However it is quite simple to detect a bad over- or under-exposure, and with a little experience it is possible to make surprisingly accurate judgment of the exposure which will be required.

LIGHT SOURCE AND DISTRIBUTION OF FILTERS

The source of light which we have used is a tungsten ribbon filament lamp, 108 watts, 6 volts, obtained from the Bausch & Lomb Optical Company. This was fitted with a lens by which the ribbon filament could be focused on the substage mirror of the microscope. As the heat from this source is sufficient to raise the temperature of the preparation above the condenser to about 55°C., producing convection currents, shrinkage

of the agar drop, and injury to the preparation it is necessary to interpose a heat absorbing chamber between the light and the mirror. For this purpose, a small museum jar with flat sides, measuring 2 by $1\frac{1}{4}$ by 4 inches, filled with water was used. The infra-red rays are effectually eliminated by this water cell.

The long exposures due to the type of shutter used in the Ciné Kodak, require great reduction of the intensity of the light. This reduced illumination is usually not sufficient for visual observation of the object through the eye-piece when all of the filter densities are placed between the light source and the substage mirror. They were, therefore, usually divided and placed in different parts of the beam of light which enters the camera. The 49B blue filter placed between the water-cell and the mirror transmits sufficient light for visual control of the focus with most preparations. The remaining density filter was fitted into the side tube of the beam-splitter at its junction with the camera-tube.

These parts of the apparatus and their arrangement are shown in plates 1 and 2 and described in the accompanying legends.

BACTERIOLOGICAL PREPARATIONS

After experiments with various types of culture-chambers and media, the most satisfactory results were obtained with the tabular hollow-ground slide illustrated in figure 2, and with clear 2 per cent agar of suitable composition for the organism to be studied. This slide is 75 mm. long, 25 mm. wide and 4 mm. thick. The concavity in the center is 14 mm. in diameter. The upper plane surface of the central table lies 2 mm. below the upper level of the slide and measures 10 mm. in diameter. It is surrounded by a moat 2 mm. across and 3 mm. deep. The culture-chamber is sterilized by passage through a flame or may be sterilized by dry heat within a receptacle, such as a Petri dish. A small agar plate is made on the top of the central table by placing melted agar on it with a sterile capillary pipette. Drops of agar are added until the convex surface of the agar is just slightly above the level of the upper surface of the slide. When the agar has cooled, a few bacteria, spores of a mold or yeast cells are

placed on its surface by means of a platinum loop. A sterile No. 1, 22-mm. square cover-glass is gently pressed down on the agar until the slide and cover-glass form a flat contact. The edges of the cover-glass are then sealed with a thin layer of vaseline, which prevents drying and holds the cover-glass in place. The moat contains sufficient oxygen for the development of aerobic organisms under the coverglass and the aerial hyphae of fungi project from the agar drop into the moat when the culture contains fungi having those forms. The problem of the proper oxygenation of the chamber, however, has not been entirely solved. Experiments are being made on a method for supplying adequate oxygen
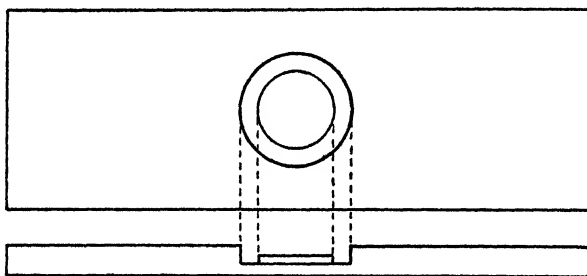
FIG. 2. MICRO-CULTURE SLIDE, SHOWING DEPRESSED CENTRAL GLASS TABLE AND MOAT

to the culture without causing drying, shrinkage and drifting of the preparation.

The organisms are seen to lie in a single plane between the agar and the cover-glass, in nutrient medium which is not distorted by shrinkage nor disturbed by flowing fluid. They grow vigorously and normally here, at room temperature, 22°C., or at 37°C. when enclosed in the usual type of simple electrically heated warm chamber designed to fit the mechanical stage of a microscope.

Films have been obtained showing the germination of single spores of a *Penicillium*, the growth and branching of mycelial filaments, and some of the phases of the development of aerial hyphae and conidiophores have been photographed by selecting special fields. The quality of the photographs, due in part to the

fine-grained image obtained when the negative is reversed to a positive on the Ciné Kodak film, permits a study of the granular structure of the interior of a mycelium. A peculiar activity and movement of granules have been observed in the film showing the growth of *Penicillium*, although this type of movement of granules could not be seen by the eye watching the growing preparation. In a film of the growth of *B. megatherium*, elongation, slight widening and transverse division of the rods are plainly shown. The extension of the sheet of single rods by crowding and pushing in this picture resembles a log-jam, and during these phases, in a film containing a record of thirty-five hours of the growth of the organisms, internal granular structure of the cells is at times sharply defined on the screen. Thousands of single organisms pass across the screen, affording material for statistical study of the size of the organisms and for studies of the morphology of the bacteria during phases of their growth.

A graphic record of the movements of *Entamoeba histolytica* has been obtained by photographing these organisms in material from a patient with amoebic dysentery. For this film, it was necessary to take the pictures at the rate of 5 per second in order to avoid blurring of the image. When the film is projected on the screen the hyaline pseudopodia, granular endoplasm, and the transport of red corpuscles ingested by the amoeba are shown distinctly.

SUMMARY

A new apparatus for motion photomicrography is described in this paper. The apparatus is relatively simple and has been constructed in part from stock materials, using the Model A Ciné Kodak to secure compactness and the advantages of the cheapness and photographic quality permitted by the 16-mm. film.[1]

[1] It is expected that the Bausch and Lomb Optical Company, Rochester, N. Y., will undertake to manufacture this apparatus Experience with the present mechanism has indicated the desirability of certain improvements in the camera-stand and cranking device, which will be embodied in the new model. Among other changes, the new form of the apparatus will contain a mechanism for making exposures at rates from one-half exposure per minute to the normal speed of 16 pictures per second in steps based upon multiples of four.

Films have been obtained with this apparatus showing the growth of *Penicillium*, *B. megatherium* and the movements of *Entamoeba histolytica*. These films provide material for scientific study and can be used in teaching bacteriology and protozoology. They indicate the suitability of the method for the general study of the growth and movements of microscopic organisms.

CHEVROTON, L., AND VLÈS, F. M. 1909 La cinématique de la segmentation de l'oeuf et la chronophotographie du dévelopment de l'oursin. C. r. l'Acad. des Sciences, Paris, **149**, 806–809

CHEVROTON, L. 1909 Dispositif pour les instantanés et la chronophotographie microscopiques. Technique de prises de vues. C. r. Soc. de Biol., Paris, **66**, 340–342

COMANDON, J. 1909 Cinématographie à l'ultramicroscope, de microbes vivant et des particules mobiles. C. r. l'Acad. des Sciences, Paris, **149**, 938–941

COMANDON, J., LEVADITI, C., AND MUTERMILCH, S. 1913 Étude de la vie et de la croissance des cellules in vitro à l'aide de l'enregistrement cinématographique. C. r. Soc. de Biol., Paris, **74**, 464–467

COMANDON, J., AND JOLLY, J. 1917–1918 Étude cinématographique de la division cellulaire. J. de Physiol. et de Path. gén., **17**, 573–589

COMANDON, J. 1920 Movements des leucocytes et quelques tactismes étudiés à l'aide de l'enregistrement cinématographique. Ann. de l'Inst. Pasteur, **34**, 1–24

COMANDON, J., ET DE FONBRUNÉ, P. 1926 Comment s'effectue la sortie de l'hémoglobine dans l'hémolyse (enregistrement cinématographique). C. r. Soc. de Biol., Paris, **95**, 635–638

FRANÇOIS-FRANCK, CH.-A. 1907 Note générale sur les prises de vues instantanées microphotographiques (plaque fixe et pellicule) avec l'arc voltaïque. C. r. Soc. de Biol., Paris, **62**, 637–638.

RIES, J. 1909 Kinematographie der Befruchtung und Zellteilung. Archiv für Mikroskop. Anat., **74**, 1–34

WEISS, M. G. 1896 Expériences dé chronophotographie microscopique. C. r. Soc. de Biol., Paris, **48**, 645–646

PLATE 1

PHOTOGRAPH OF LATERAL VIEW OF APPARATUS

1. View-finder.
2. Flanged joint for contact with camera tube.
3. Microscope with mechanical stage and culture slide.
4. Model (A) Ciné Kodak.
5. Adjustable camera stand with motor.
6. Control board with: 7, rheostat; 8, switch for motor and 9, light switch

(Bayne-Jones and Tuttle Apparatus for Motion Photomicrography)

171
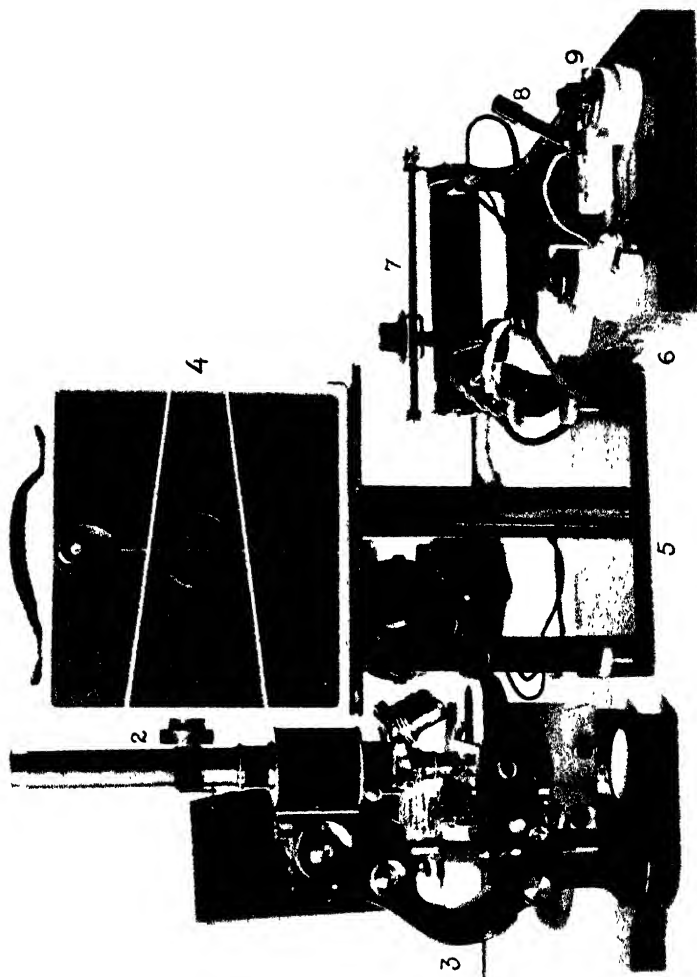
PLATE 2

PHOTOGRAPH OF ANTERO-LATERAL VIEW OF APPATATUS

1 View-finder.
2 Flanged joint for contact with camera tube, slip ring disengaged.
3. Microscope.
4. Water cell
5 Holder for light-filters
6 Lamp, ribbon filament 6 volts, 108 watt.
7. Model (A) Ciné Kodak
8 Gear box attached to camera-crank.
9 Motor and pulleys, with coiled spring belt.
10. Adjustable camera stand.

PLATE 2
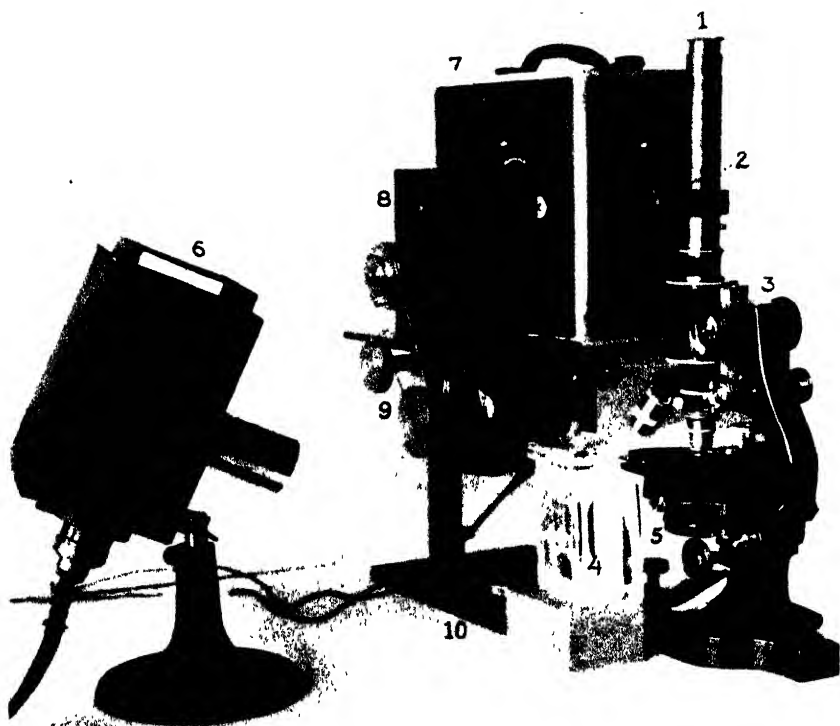
(Bayne-Jones and Tuttle Apparatus for Motion Photomicrography)

A COMPLETE DESCRIPTION OF CLOSTRIDIUM PUTREFACIENS (McBRYDE)

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More than fifteen years ago McBryde (1911) isolated an interesting anaerobe differing in several characters from any previously described. He suggested the name *B. putrefaciens* for this new species. Although a considerable description of this organism, based presumably on a single culture, was given in this bulletin, it seems not to have received any recognition in the literature of the subsequent fifteen years. Recently Boyer (1926) reports the frequent finding of this organism, and also of other anaerobes, in animal muscle tissue soon after slaughter.

In this Laboratory during the past few years, 27 strains have been isolated from animal sources which conform with the anaerobe already described. They are strict anaerobes of the clostridial type and are markedly proteolytic. Young cultures in meat media are characterized by filaments and chains. After two or three days cultivation the cells become club-shaped, this deep-staining terminal swelling being best described as bulbed. From this initial stage sporulation proceeds rapidly and mature spores are found a day or two later. They are plectridial, being terminal, spherical, and about 2.2 times the diameter of the rod. The typical drumstick sporangia remain intact for several weeks, during which time free spores are extremely rare. When found these are faintly stained (Gram method) and are apparently thin-walled. Very old cultures show considerable disintegration of cells with the liberation of many of these faint-staining free spores.

While this is the typical behavior of *C. putrefaciens* when first

cultured in the laboratory, it must be admitted that the writers in common with Boyer (1926) have noted a considerable lessening of sporulation after prolonged cultivation of pure cultures. No other changes in the behavior have been observed.

A detailed study of these 27 strains based on the latest S. A. B. Descriptive Chart has been made and is now offered in confirmation of the original work of McBryde. The various refinements of methods which have developed within the past fifteen years have

FIG. 1. CLOSTRIDIUM PUTREFACIENS OLD CULTURE IN PORK MEDIUM WITH AN INITIAL pH OF 8.0 × 1200

been employed in our attempt to establish this unique organism among the anaerobic clostridia where it undoubtedly belongs.

Morphology. Rods 0.5–0.7 by 3–15μ with rounded ends, occurring singly and in chains. Drumstick sporangia with spherical terminal spores 1.1–1.6μ in diameter. Non motile. Gram positive. Thick filaments predominate in low pH media.

Agar colonies. Small, filamentous.

Agar slant. Scanty, white, beaded, glistening.

Broth. Moderate, transient, turbidity; heavy growth occurring as a flocculent sediment at bottom.

Minced pork. Slight disintegration of meat particles with strong, characteristic, sour, putrefactive odor. There is little visual evidence of digestion of the meat until the tube is shaken vigorously when the contents disintegrate into a more or less homogeneous pulp.

Gelatin stab. Growth and liquefaction starting at bottom in two to three days. Liquefaction complete in three to five days.

Litmus milk. Litmus reduced in four to seven days. Rennet curd appears in fifteen days at a pH of 6.5. Peptonization follows slowly.

Indol not formed.

Hydrogen sulphide. Slight production.

Nitrates not reduced.

Fermentation. Acid and slight gas in glucose. No acid or gas in lactose, sucrose or maltose. Starch not hydrolyzed.

Anaerobic. No surface growth on any medium without strict anaerobiosis.

Temperature relations. Optimum 20° to 25°C. Slow growth at 0°C. and even at lower temperatures. No visible growth at 37°C.

pH relations. Growth occurs in meat medium between pH 6.0 and pH 9.0 with an optimum at pH 8.0.

Habitat. Found in muscle tissues of hogs at slaughter.

In common with McBryde we have found pork extract to be favorable for growth, and it has been used as the basis of the agar, gelatin, and broth media employed. Anaerobic conditions were obtained by twice evacuating to the vapor pressure of water and refilling with hydrogen.

It will be noted that our negative findings for indol are at variance with McBryde's. Cultures in both pork extract pepton broth and tryptophane broth (Bacto) have been tested at the ages of one, three and seven days by the Ehrlich test. A slight positive test was obtained when the cultures were tested directly. It was found, however, that the color could not be extracted with chloroform. Duplicate cultures were subjected to a steam distillation according to the technique of Fellers and Clough (1925). No positive test could be obtained by this method.

From the foregoing study it is evident that *C. putrefaciens* is a most interesting anaerobe, so far as the writers are aware

unique in its temperature requirements. This strongly proteo-
lytic organism grows well at 0°C. and little or not at all at 37°C.

Morphologically the typical drumstick sporangia are sugges-
tive of *C. putrificum* (Bienstock) and *C. tetani*. The rods of *C.
putrefaciens*, however, have a slightly greater diameter than those
of *C. putrificum* and often exceed those of *C. tetani* in length.
Furthermore the lack of motility and the low temperature re-
quirements of the first species differentiate it from the two latter.
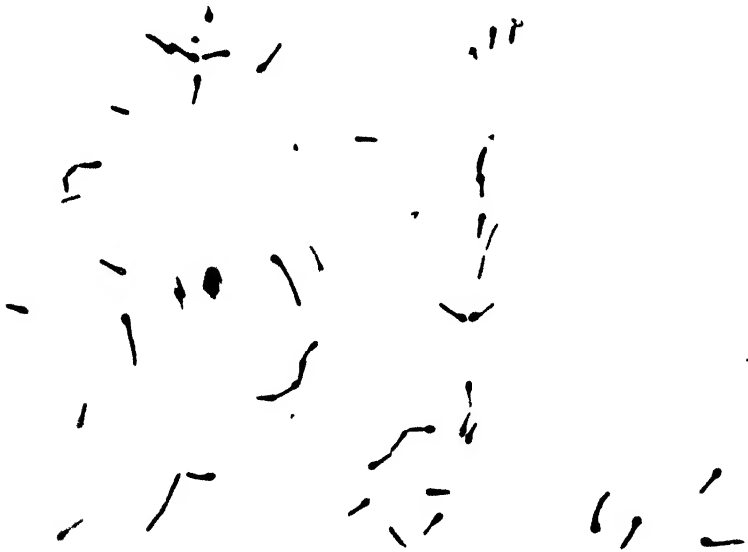
Fig. 2 CLOSTRIDIUM PUTREFACIENS GROWN TEN DAYS IN PORK MEDIUM WITH
AN INITIAL pH OF 6.8. × 1200.

The odor produced in meat is very characteristic. One accus-
tomed to these odors has no difficulty in differentiating pure
cultures of this anaerobe from other putrefactive organisms by
this means.

Old meat cultures of *C. putrefaciens* present a sharp contrast
with similar cultures of other putrefactive anaerobes in which
extensive liquefaction is perfectly evident. A marked softening
of the meat without evident reduction in bulk is characteristic of
C. putrefaciens.

The twenty-seven strains studied, although isolated from different sources, show perfect agreement on all characteristics. This agreement in cultural and physiological details, the consistently characteristic and distinctive microscopic picture, and our repeated failure to obtain growth from any of the strains at 37° under anaerobic conditions, or at any temperature under aerobic conditions, constitute a part of our criteria for considering these cultures to be pure.

The writers consider this unique organism deserving of recognition as a distinct species and suggest its inclusion in the genus Clostridium, as *C. putrefaciens*.

REFERENCES

BOYER, E. A.   1926   Jour. Agri. Res., **33**, 761.
FELLERS, C. R., AND CLOUGH, R. W.   1925   Jour. Bacteriol., **10**, 105–133.
MCBRYDE, C. N.   1911   B. A. I. Bull. 132 U. S. Dept. Agric.

QUANTITATIVE ASPECTS OF THE METABOLISM OF ANAEROBES

I. PROTEOLYSIS BY CLOSTRIDIUM PUTREFACIENS COMPARED WITH THAT OF OTHER ANAEROBES

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A series of studies concerning various quantitative aspects of the metabolism of the anaerobes is being carried out in this laboratory. This paper, the first of the series, presents data demonstrating the proteolytic activity of *C. putrefaciens*, an organism originally isolated and described by McBryde (1911). A complete description of this anaerobe has been published in the preceding paper by Sturges and Drake. No definite quantitative information has been available concerning its powers of protein decomposition. It is, therefore, deemed desirable to record some data permitting a comparison with several common putrefactive anaerobes.

Twenty-four strains of *C. putrefaciens* isolated from various sources were investigated.

Nutrient gelatin (Bacto-dehydrated) without sugar, and cooked meat media were used. While heterogeneous media of various types are well suited for the cultivation of the anaerobes, difficulties arise in securing representative samples. The sampling difficulty has been obviated by weighing out definite quantities of finely ground, well mixed, lean meat into large tubes, adding buffer, mixing and sterilizing. Analyses for total nitrogen made on the ground meat give a basis for computation. After inoculation and incubation the entire contents of the tube are used for analysis.

The exact procedure has been to weigh 25 grams of ground lean

TABLE 1

Influence of pH on the growth and morphology of C. putrefaciens

AGE (days)	OBSERVATION OF	GROWTH IN MEDIA AT VARIOUS INITIAL pH VALUES									
0	Initial pH	5.80	6 15	6 26	6 45	6 61	6 90	7 70	8 30	8 60	8 98
	Formol N	137	136	146	153	153	157	162	165	166	167
6	Observed pH	–	6.04	6 15	6.27	6 43	6 50	6 62	6 80	7 02	7 09
	Formol change	65	154	218	325	399	477	636	639	557	386
	Width	T	T	T	M	M	M	M–Tn	M–Tn	M–Tn	Tn
	Length	L	L	L	L–S	M–S	M–S	M–S	M–S	M–S	M–S
	Spores	–	–	–	B	–	B	B	B	B	–
	Chains	–	–	–	F	F	M	M	M	M	–
	Filaments	M	M	M*	F	F	–	–	–	–	–
10	Observed pH	5.75	6 55	–	–	6 60	6 65	6 70	7 10	7 05	7 15
	Formol change	70	235	345	409	497	669	720	790	660	476
	Width	T	M	M	M	M	M	M–Tn	M–Tn	M–Tn	M–Tn
	Length	L	L–S	L–S	M–S	M–S	S	S	S	S	M–S
	Spores	B	–	F	B	B	B+	B+	B+	B+	–
	Chains	–	F	F	F	F	M	M	M	M	M
	Filaments	M	M	–	F	F	–	–	–	–	–
27	Observed pH	5 95	6 35	6 45	6.55	6.70	6 85	6 95	7.00	–	7 35
	Formol change	122	374	604	667	737	918	973	973	850	780
	Width	T	T	T	T	T	M	M	M–Tn		M–Tn
	Length	L	L–S	S	S	S	S	S	S		M–S
	Spores	–	–	B	B	B	B	B+	B+		B+
	Chains	–	–	F	F	F	M	M	M		M
	Filaments	M	F	–	–	–	–	–	–		F

60	Observed pH	6.00	6 20	6.70	6.80	6 60	6 70	7.20	7.20	7.50	7.50
	Formol change	192	575	740	865	929	1043	1096	1126	922	803
	Width		T	T	T	T	T-M	M	M	M-Tn	Tn
	Length		L	L	S	S	S	S	S	S	M
	Spores		—	—	B	B+	B+	B+	B	—	—
	Chains		—	F	F	F	M	M	M	M	M
	Filaments		—	—	—	—	—	—	—	—	—

Width: T = thick; M = medium; Tn = thin.
Length: L = long; M = medium; S = short.
Spores: B = bulbs only; + = spores present.
Chains: F = few; M = many.
Filaments: F = few; M = many.
* See microphotograph (fig. 1).

pork into a 1- by 8-inch tube. To this is added 20 cc. of a sodium hydroxide-phosphate buffer (125 cc. molar sodium hydroxide + 100 cc. molar disodium phosphate diluted to a liter). The meat and buffer are thoroughly mixed by means of a thin glass stirring rod. After sterilization a pH of 7.5 to 8.0 is obtained. A number of analyses on various samples of freshly ground lean pork tissue
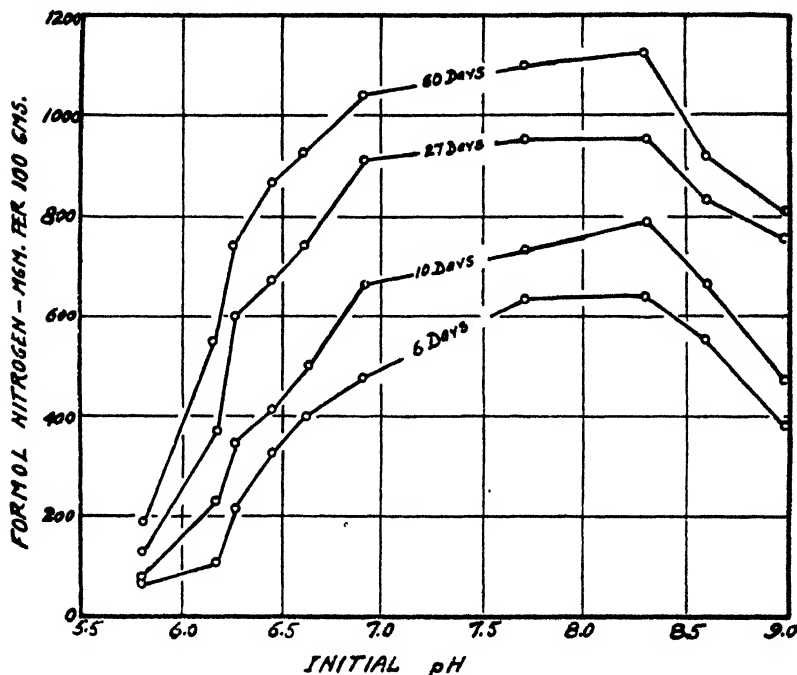
CHART 1. THE GROWTH OF C. PUTREFACIENS IN MEAT MEDIUM AT 20°C. AS A FUNCTION OF INITIAL pH (REGISTERED BY PRODUCTION OF FORMOL NITROGEN)

gave 2900 to 3000 mgm. N per 100 grams. Because of the small difference in the analytical results the average value of 2950 mgm. N per 100 grams has been used for computations.

The quantitative determination of ammonia was by the Folin aeration procedure, and amino nitrogen was determined by the formol titration according to J. H. Brown (1923). The gelatin medium was used directly for analysis. The meat medium was prepared for analysis by washing the contents of the tube into

a 250 cc. volumetric flask. The tissue residue was broken up to allow complete extraction, and finally the volume was made to the mark and thoroughly mixed. The mixture was filtered through a dry folded filter paper into a dry flask and definite portions of the filtrate were used for analysis. It is obvious that 1 cc. of the filtrate corresponds to 0.1 gram of meat. In the analytical determinations controls were invariably run on the medium, using the same sized samples that were used for estimation on the cultures.

FIG 1 CLOSTRIDIUM PUTREFACIENS GROWN SIX DAYS IN PORK MEDIUM WITH AN INITIAL pH OF 6 26 X 1200

These controls have been subtracted from the observed figures to give the results reported in this paper.

The gelatin cultures were incubated in an atmosphere of hydrogen. A thorough anaerobiosis was insured by exhaustion with a Cenco Hi-Vac pump, filling with electrolytic hydrogen, re-exhausting and refilling. In the meat medium described in this paper uniformly satisfactory growth occurred without resorting to anaerobic methods.

The optimum hydrogen ion concentration and the effect of

variations in initial pH of the medium upon the morphology of a representative strain of *C. putrefaciens* was determined. The quantitative meat medium was adjusted to pH values ranging from 5.8 to 9.0, inoculated and incubated at 20°C. for varying periods. The change in formol nitrogen was taken as an index of the extent of growth; microscopic observations were made on
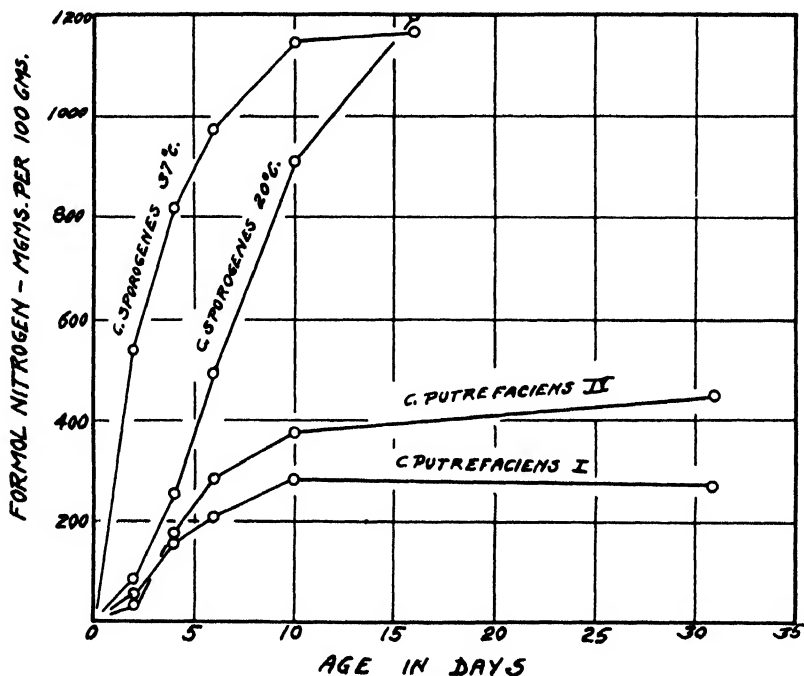
CHART 2. THE RANGE OF FORMOL CHANGE PRODUCED IN GELATIN MEDIUM BY VARIOUS STRAINS OF C. PUTREFACIENS AT 20°C. CONTRASTED WITH C. SPOROGENES

the various cultures at the same time. The data are presented in table 1 and chart 1.

It will be seen from chart 1 that the optimum initial pH for this organism is 8.0 ± 0.3. The zone for good growth is fairly wide however, and it is to be noted that the influence of initial pH is less marked in the older cultures. When growth is once initiated the large quantities of ammonia and volatile acids

produced tend to lower the pH of the high pH medium rapidly, and to raise the pH of the low pH medium. In table 1 the sets of cultures close to the optimum initial pH gave final values of 7.0 to 7.2. The optimum value obtained has been found useful in that all strains of *C. putrefaciens* have given uniformly good growth in media of initial pH 7.8 to 8.0.
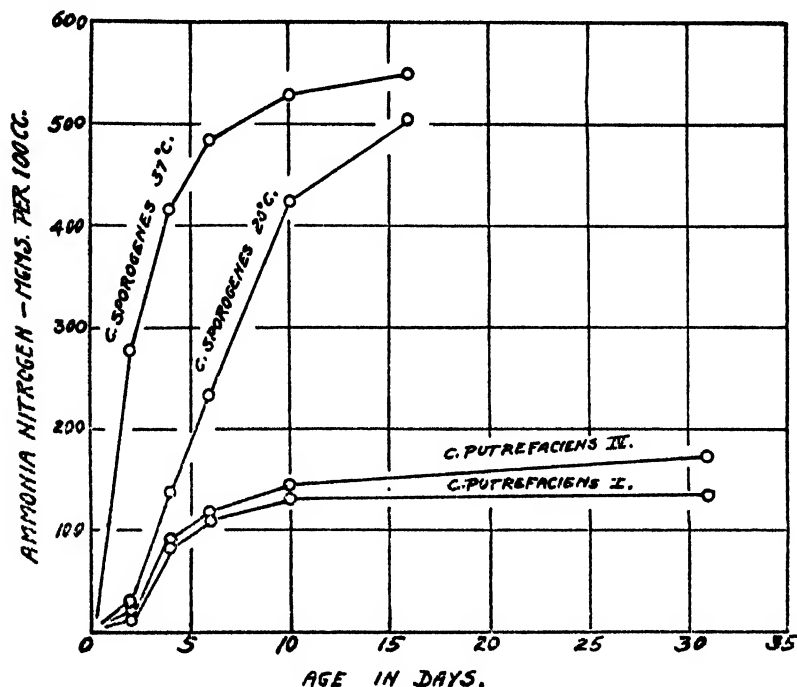
CHART 3. THE RANGE OF AMMONIA CHANGE PRODUCED AT VARIOUS STRAINS OF C. PUTREFACIENS IN GELATIN MEDIUM AT 20°C. CONTRASTED WITH C. SPOROGENES

The microscopic picture reported in table 1 shows to an astonishing degree the influence of pH on morphology. For example, the characters of the organisms at six days in a medium of initial pH 6.26 were in such marked contrast to those of pH 7.70 of the same age that one would scarcely have recognized them as the same species. The former consisted of long thick rods and many thick filaments, while the latter showed medium to thin

rods of medium to short length with many chains and some bulbs representing the early stages of sporulation.

We shall present the quantitative data on protein decomposition in graphic form, since we believe that masses of data are thus more easily grasped and that there is less tendency to over-emphasize insignificant differences.
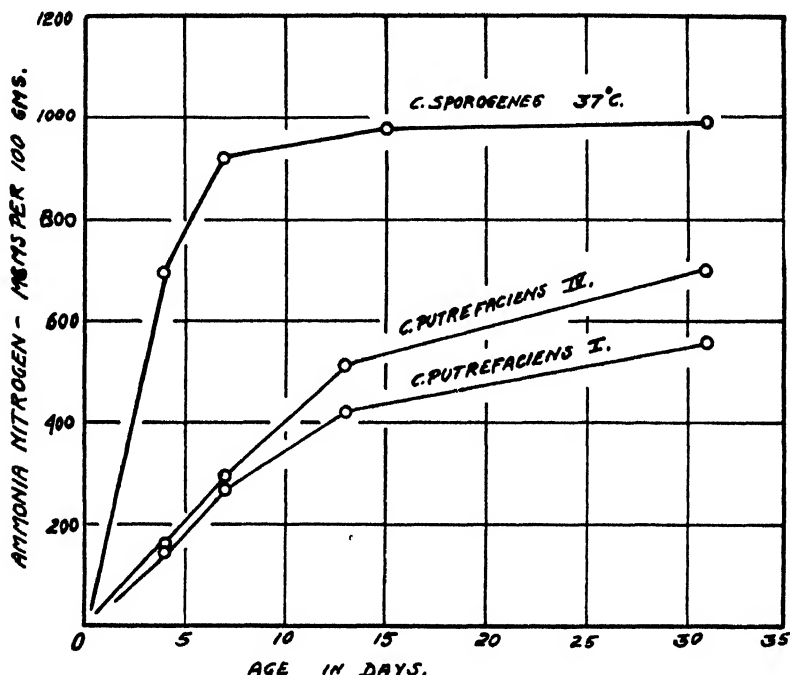
CHART 4.   THE RANGE OF PRODUCTION BY TWO STRAINS OF C. PUTREFACIENS IN MEAT AT 20°C. CONTRASTED WITH C. SPOROGENES

The proteolytic cleavage of gelatin by the first six strains of *C. putrefaciens* isolated was studied at 20°C.   The rate of increase in formol titration for strains I and IV, respectively the least and most active cultures, are plotted in chart 2.   For comparison similar results with *C. sporogenes* in the same medium incubated at 20° and 37°C. are included.   In chart 3 similar comparisons of ammonia production are given.   From these charts it is evident that the rate of production of formol and ammonia nitrogen is

slower for *C. putrefaciens* than for *C. sporogenes* and that the maximum conversion is only about one-third as much.

We have plotted data on *C. sporogenes* at the optimum temperature of 37°C. and also at 20°C. This is pertinent because it demonstrates that the only effect of decreasing the temperature by this amount is to decrease the rate, with no apparent effect
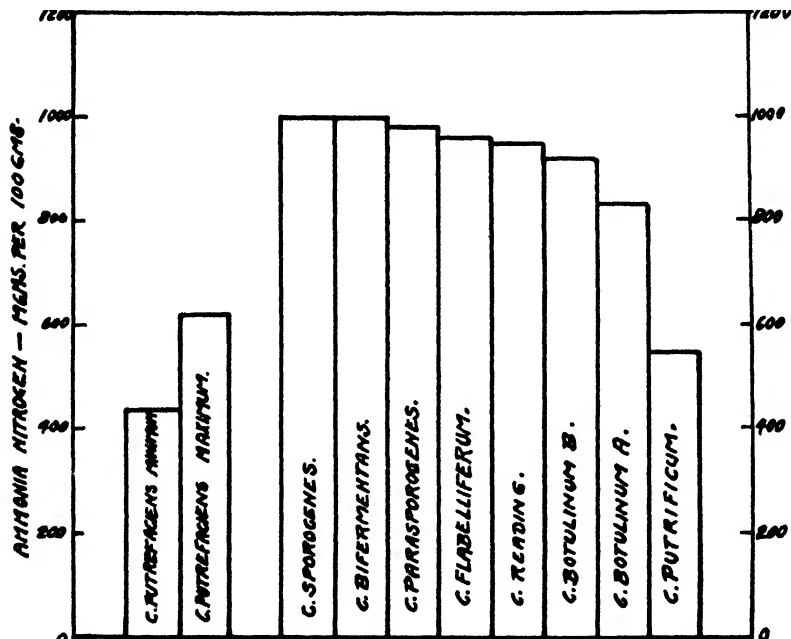
CHART 5. COMPARISON OF THE PROTEOLYTIC ACTIVITY OF C. PUTREFACIENS IN TWELVE-DAY MEAT CULTURES WITH THAT OF SOME COMMON PUTREFACTIVE ANAEROBES

on the final result. Subsequent comparisons have been made using the optimum temperatures of 37° for the other anaerobes and 20° for *C. putrefaciens*.

Chart 4 gives data for ammonia production in meat medium. No figures on formol nitrogen for other anaerobes were available. This chart shows *C. putrefaciens* to produce about 0.8 as much ammonia in meat medium as *C. sporogenes*. It should be noted that in subsequent observations of twenty-four strains of *C.*

putrefaciens 775 mgm. of ammonia N was the maximum obtained up to an age of forty days.

Chart 5 is a comparison of ammonia production by twenty-four strains of *C. putrefaciens* in twelve-day-old meat culture with cultures of some of the common anaerobes of the same age. This chart suggests that *C. putrefaciens* is somewhat less active than the other anaerobes. As noted above the highest ammonia production obtained (775 mgm. per 100 grams) in meat does not give a wide margin of difference.

C. putrificum (Bienstock) with 550 mgm. ammonia N per 100 grams might seem to be of about the same activity as *C. putrefaciens*. In reality it is extremely proteolytic, producing in this experiment 1300 mgm. of ammonia N in thirty-eight days, which is in marked contrast with *C. putrefaciens* which never seems to form more than 775 mgm.

Cultures of *C. putrefaciens* have a distinctly sour, characteristic odor with little suggestion of mercaptans and hydrogen sulfide. Estimations of hydrogen sulfide were made by the method of Fellers, Shostrom, and Clark (1924). Cultures of *C. putrefaciens* and *C. sporogenes* were grown in quantitative meat medium (5 grams of meat + 4 cc. buffer) in evacuated tubes. After suitable incubation the tubes were broken under caustic soda solution in the aeration cylinder, finally acidified and aerated. This modification was made to avoid possible loss of hydrogen sulfide during manipulation. After 12 days incubation duplicate determinations on *C. sporogenes* gave 37.0 and 37.5 mgm. H_2S per 100 grams of meat, while four determinations on *C. putrefaciens* yielded 7.5, 7.7, 8.7, and 8.2 mgm. per 100 grams respectively.

DISCUSSION

Boyer (1926), referring to *C. putrefaciens*, states, "This organism is primarily of the saccharolytic group and gives no evidence of digestion in cooked meat medium." This statement we assume is based entirely on visual evidence since there is, at most, slight apparent digestion of the meat. However, it is so softened that it easily disintegrates on agitation. The data presented in this paper definitely establish this anaerobe in the proteolytic

group, since it is obvious that an organism which converts 30 to 40 per cent of the total nitrogen in meat to amino acids and ammonia, cannot be held to be primarily saccharolytic.

The fact that *C. putrefaciens* produces in meat about eight-tenths and in gelatin about three-tenths as much ammonia as *C. sporogenes* suggests a fundamental difference between the two organisms. The writers feel that quantitative differences in the products of proteolytic changes have been very often over emphasized in the identification of anaerobes. Aside from the fact that different strains of the same species exhibit considerable differences in activity under identical conditions, it still remains problematical whether various investigators working with the same culture can obtain identical results. For example, in chart 5 the differences in the amounts of ammonia produced by the common anaerobes are doubtless insignificant, while the differences between them and *C. putrefaciens* and *C. putrificum* appear to be perfectly definite. It has already been noted that *C. putrificum* grows slowly but finally produces much larger amounts of ammonia than *C. putrefaciens*. If the sole claim for the recognition of the latter organism as a definite entity were based on these differences, we should hesitate to recognize it as a distinct species. We shall, however, in other communications present further evidence to justify the recognition of this unique organism.

The data presented on hydrogen sulfide seem to explain in part the failure of *C. putrefaciens* to produce the typical odors obtained from cultures of the other putrefactive anaerobes in which the volatile sulfides predominate. The relatively small hydrogen sulfide production appears to be a point of distinction between this organism and *C. sporogenes*.

SUMMARY

The pH optimum for *C. putrefaciens* has been determined and its marked effect on morphology noted. This anaerobe has been shown to be decidedly proteolytic in gelatin and cooked meat medium, and a quantitative comparison has been made with other anaerobes.

C. sporogenes has been found to produce about five times as much hydrogen sulfide as *C. putrefaciens*.

REFERENCES

BOYER, E. A. 1926 Jour. Agri. Research, **33**, 761.
BROWN, J. H. 1923 Jour. Bacteriol., **8**, 245.
FELLERS, C. R., SHOSTROM, O. E., AND CLARK, E. D. 1924 Jour. Bacteriol., **9**, 235.
MCBRYDE, C. N. 1911 U. S. Dept. Agri., B. A. I. Bull. 132.

QUANTITATIVE ASPECTS OF THE METABOLISM OF ANAEROBES

II. THE RELATION BETWEEN VOLATILE ACID AND AMMONIA PRODUCTION DURING METABOLISM OF CLOSTRIDIUM PUTREFACIENS

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Several investigators have quantitatively measured organic acids produced during the protein metabolism of the anaerobes, but no systematic study of the significance of these acids has been reported. Wagner, Dozier, and Meyer (1924) state that in a general way volatile acid production parallels ammonia accumulation for *C. botulinum, C. tetani* and *C. sporogenes,* and these same investigators (1925) draw "derived curves" showing among other things the relationship of these products to each other. In so far as the writers are aware, no one has ever shown any quantitative relationship to exist between ammonia and volatile acid production by the putrefactive anaerobes.

Protein degradation by the anaerobes probably takes place by the exogenous, enzymatic hydrolysis of protein to polypeptides. Whether or not all the amino acids produced are formed exogenously seems to be a matter of conjecture. There appears to be no satisfactory evidence that ammonia is ever formed by exogenous enzymes. Kendall and Walker (1915), and Kendall (1921), regard ammonia formation as an exact measure of the intracellular utilization of amino acids or their complexes for bacterial energy. Concomitantly with the formation of ammonia a considerable quantity of acid is produced and carbon dioxide is evolved. These latter products appear to result from the deaminization of the amino acids by some unknown mechanism and the whole process may be the source of bacterial energy.

It would seem that two possible mechanisms for the deaminization of amino acids by the anaerobes may be formulated.

(1) $R \cdot CHNH_2COOH + H_2O = R \cdot CHOH \cdot COONH_4$
(2) $R \cdot CHNH_2 \cdot COOH + H_2 = R \cdot CH_2 \cdot COONH_4$
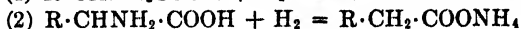
The hydrolytic deaminization sketched in equation 1 would result in the formation of hydroxy acids (lactic, etc.) while the reductive deaminization equation 2 would result in the formation of saturated acids (acetic, etc.). The mechanism, of course, is not as simple as sketched and there is no *a priori* reason why (1) and (2) might not proceed simultaneously nor why carbon dioxide might not be evolved by some oxidative mechanism. From the above considerations we thought it likely that some definite molecular relationships might exist between the acids and ammonia produced by putrefactive anaerobes. Data which we are presenting in this communication definitely establish a molecular equivalence between the ammonia and volatile acids produced by *C. putrefaciens* in both meat and gelatin media at all stages of growth.

The media used were 10 per cent nutrient gelatin (Difco) without sugar, and the quantitative meat medium described in the first paper of this series.

Ammonia was determined by the aeration procedure of Folin, using saturated sodium carbonate solution to liberate the ammonia. Volatile acids were determined by steam-distilling acidified samples in a special distillation apparatus described elsewhere. The distillate was titrated with $N/10$ sodium hydroxide. The solutions used were frequently re-standardized to insure maximum accuracy. Volatile acid found has been reported in cubic centimeters of $N/10$ acid per 100 grams or 100 cc. To furnish a molecular basis of comparison, the number of milligrams of ammonia nitrogen is divided by 1.4 to convert to cubic centimeters of $N/10$ ammonia. Suitable controls have invariably been subtracted from all results, the data reported being net changes in ammonia and volatile acid.

The twenty-four strains of *C. putrefaciens* used in Paper I of this series were used in these investigations. Incubation was at 20°C.

Table 1 presents the data on five strains of *C. putrefaciens* in gelatin medium. As a criterion of equivalence the last column

<div align="center">TABLE 1</div>

Volatile acid—ammonia relations for C. putrefaciens in gelatin

STRAIN	AGE	NH₃N PER 100 CC.	N/10 NH₃ PER 100 CC.	N/10 V.A. PER 100 CC.	V.A.-NH₃ RATIO
	days	*mgm.*	*cc.*	*cc.*	
	2	29	21	27	1 28
	4	80	57	57	1 00
	6	101	72	72	1 00
C. putrefaciens I.............	10	132	94	87	0 93
	16	149	106	101	0.95
	31	137	98	94	0.96
	2	21	15	16	1.07
	4	49	35	36	1 03
	6	90	64	65	1.02
C. putrefaciens II....	10	148	106	105	0 99
	16	178	127	121	0 95
	31	167	119	114	0.96
	2	12	9	9	1.00
	4	52	37	37	1 00
	6	80	57	62	1 09
C. putrefaciens III...........	10	172	123	125	1 02
	16	152	109	108	0.99
	31	190	136	128	0.94
	2	11	8	9	1.13
	4	72	52	53	1.02
	6	106	75	75	1 00
C. putrefaciens IV.	10	143	102	104	1 02
	16	156	111	110	0.99
	31	172	123	118	0.96
	2	13	9	11	1.22
	4	63	45	48	1.07
	6	103	73	73	1.00
C. putrefaciens VI...........	10	136	97	98	1.01
	16	161	115	113	0.98
	31	169	121	120	0.99

gives the ratio of moles of volatile acid to moles of ammonia. We have termed this the V.A.–NH₃ ratio. The ratios for the two

day old cultures appear to be far from unity, but it must be remembered that the experiments were performed on separate tubes and that small initial differences, as well as slight errors in analysis are greatly magnified in this ratio.

TABLE 2

Volatile acid—ammonia relations for C. putrefaciens in meat

STRAIN	AGE	NH₃N PER 100 GRAMS	N/10 NH₃ PER 100 GRAMS	N/10 V.A. PER 100 GRAMS	V.A.-NH₃ RATIO
	days	*mgm.*	*cc.*	*cc.*	
	4	168	120	121	1.01
	6	311	222	226	1.02
C. putrefaciens I	10	396	283	294	1.04
	16	443	316	335	1.06
	30	558	398	408	1.03
	4	216	154	150	0.97
	6	340	243	238	0.98
C. putrefaciens II	10	389	279	286	1 03
	16	559	399	404	1.01
	30	676	483	467	0.97
	4	240	171	166	0.97
	6	379	271	272	1 00
C. putrefaciens III	‘ 10	450	322	332	1 03
	16	500	357	360	1.01
	30	668	477	492	1.03
	4	207	148	149	1 01
	6	317	226	226	1.00
C. putrefaciens IV	10	449	321	332	1.03
	16	468	335	345	1.03
	30	610	435	447	1.03
	4	254	181	176	0.97
	6	357	255	255	1.00
C. putrefaciens VI	10	358	256	259	1.01
	16	534	382	381	1.00
	30	708	506	527	1.04

In table 2 figures are given for the same cultures in quantitative meat medium at various ages. In table 3 data are presented demonstrating that the V.A.-NH₃ ratio is unity for all strains isolated. Two tubes of each strain were grown. Duplicate analy-

TABLE 3

Volatile acid—ammonia relations for all strains of C. putrefaciens in meat at 12 days

STRAIN NUMBER	NH₃N	N/10 NH₃	N/10 V.A.	V.A.–NH₃ RATIO
	mgm.	*cc.*	*cc.*	
1	491	350	369	1.06
1	492	351	358	1.02
2	506	362	406	1.12
2	565	404	415	1.03
3	486	347	363	1.05
3	558	398	405	1 02
4	516	368	405	1.10
4	567	405	413	1.02
5	521	372	388	1.04
5	565	407	417	1.03
6	528	378	388	1.03
6	608	434	443	1 04
7	438	313	330	1.05
7	479	342	330	0.97
8	497	356	379	1 06
8	561	401	409	1.02
9	530	378	398	1.05
9	596	426	425	1 00
10	489	350	370	1.06
10	566	405	417	1.03
11	541	386	367	0.95
11	581	415	405	0.98
12	526	376	381	1.01
12	552	395	390	0.99
14	446	318	322	1.01
14	478	342	336	0.98
15	503	360	362	1.00
15	540	386	386	1.00
16	435	311	325	1.04
16	456	326	339	1.04
17	484	346	354	1.02
17	465	332	344	1.04
18	471	336	341	1.01
18	522	374	378	1.01
19	479	342	352	1.03
19	496	354	353	1.00
20	491	350	365	1.04
20	569	406	420	1.03
21	495	354	360	1.02
21	532	380	379	1.00
22	527	377	381	1.01
22	560	400	388	0.97
23	487	348	350	1.01
23	512	368	370	1.01
24	569	407	412	1.01
24	522	372	371	1.00

ses for volatile acid and ammonia were made on each tube and averaged to give the results in this table. It has also been found that media made of minced pork heart or of beef tissue give V.A.–NH$_3$ ratio of unity.

Since the data clearly indicated that volatile acid and ammonia were formed mole for mole it became of interest to determine whether any non-volatile (fixed) acids were produced. We have determined the total acids in the sample by extracting the acidified deproteinated material with ether in a liquid extraction apparatus. The extract was placed in a large beaker with 400 cc. of water and the ether evaporated spontaneously. The acid was

TABLE 4

Comparison between total acid and volatile acid produced by C. putrefaciens in meat at twenty-five days

CULTURE	TOTAL ACID N/10 PER 100 GRAMS	VOLATILE ACID N/10 PER 100 GRAMS	NON-VOLATILE ACID N/10 PER 100 GRAMS	V.A.–NH$_3$ RATIO
	cc.	cc.	cc.	
C. putrefaciens 1............................	484	480	+4	1.01
C. putrefaciens 6	513	511	+2	1.06
C. putrefaciens 8............................	540	544	−4	1.00
C. putrefaciens 9............................	475	480	−5	0.99
C. putrefaciens 10...........................	490	502	−12	0.99
C. putrefaciens 16...........................	499	494	+5	1.01
C. putrefaciens 18...........................	538	541	−3	1.04
C. putrefaciens 19...........................	571	561	+10	1.01

then titrated with N/10 caustic. Suitable controls were run on uninoculated media and subtracted to give the values reported. The procedure was tested on a mixture consisting of 13.05 cc. N/10 acetic acid and 9.35 cc. of N/10 lactic acid in a volume of 25 cc. After sixteen hours extraction a total titration of 22.80 cc. was obtained compared with 22.40 taken. All extractions were run twenty hours using a volume of 25 cc. of liquid. The difference between the total acid and volatile acid is, of course, non-volatile acid. Table 4 gives the data obtained on the several strains which have been tested.

It is quite evident from the above table that *C. putrefaciens*

does not produce any non-volatile acid in meat medium. No measurements have been made in gelatin medium.

Tables 1, 2 and 3 contain data which conclusively prove that *C. putrefaciens* in gelatin and meat produces one molecule of volatile acid for each molecule of ammonia resulting from deamidization processes. Moreover, table 4 shows that this organifm produces no nonvolatile acid in meat medium and we may possibly infer that the same is true in gelatin. These facts seem to furnish definite evidence that a reductive deamidization such as represented in equation 2 is taking place. *C. putrefaciens* in common with the other putrefactive anaerobes produces carbon dioxide from both gelatin and meat. Preliminary experiments in which this organism was grown in vacuo and the total carbon dioxide produced was measured by acidifying the sample, with subsequent removal and analysis of the gas, have demonstrated that approximately one mole of carbon dioxide for three moles of ammonia results from growth in these media. Evidently the reductive deamidization is complicated by a simultaneous or subsequent process yielding carbon dioxide.

Gelatin and meat are notably different in the amino acids yielded by complete hydrolysis, the former being characterized by small amounts of dibasic mono amino acids while the latter yields relative large quantities of these acids. Since *C. putrefaciens* gives the same V.A.–NH_3 ratio in two such widely different media, the deamidization and CO_2 producing mechanism would appear to be highly specific suggesting that only certain amino acids can be attacked. It is futile to speculate further at this time although it is believed that these results indicate a new line of attack on the fundamental problem of protein metabolism.

SUMMARY

Volatile acid and ammonia production by *C. putrefaciens* has been quantitatively determined in meat and gelatin media at various intervals.

The molar ratio of volatile acid to ammonia was found to be unity indicating an equivalence between these products at all ages.

Non-volatile acid is not produced by *C. putrefaciens* in meat medium.

The results suggest a high degree of specificity in the deaminization of amino acids by this organism.

REFERENCES

KENDALL, A. I. 1922 Jour. Inf. Dis., **30**, 211.
KENDALL, A. I., AND WALKER, A. W. 1915 Jour. Inf. Dis., **17**, 442.
WAGNER, E. DOZIER, C. C., AND MEYER, K. F. 1924 Jour. Inf. Dis., **34**, 63.
WAGNER, E., DOZIER, C. C., AND MEYER, K. F. 1925 Jour. Bacteriol., **10**, 321.

QUANTITATIVE ASPECTS OF THE METABOLISM OF ANAEROBES

III. THE VOLATILE ACIDS PRODUCED BY C. PUTREFACIENS IN COOKED MEAT MEDIUM

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A limited amount of information concerning the nature of the volatile acids produced by a few anaerobes is to be found in the literature. For the most part these investigations have been incidental to other phases of anaerobic metabolism and are inconclusive, due either to scanty data or inadequate methods.

Seliber (1914), using the Duclaux (1900) method, reported difficulty in determining the nature of the acids produced by *C. putrificum*. Wolf and Telfer (1917) used a modified Dyer (1916) technique to estimate the acids produced by *C. sporogenes* in milk, while Bushnell recorded figures for steam distillation of the volatile acids produced by this organism in several media. Wagner, Dozier and Meyer (1924) (1925) have presented data on *C. tetani*, *C. sporogenes* and *C. botulinum* in beef heart tryptic digest medium. These investigators used both the Dyer and Duclaux methods in their studies, the former method to obtain an approximate idea of the nature of the acids present and the latter to estimate quantitatively the individual acids in the mixture. They report that old cultures of *C. sporogenes* and *C. botulinum* contain a larger proportion of high molecular weight acid than young cultures, while *C. tetani* gives the same acids in the same proportions in young and old cultures. Bushnell's (1922) data show no consistent variations from young to old cultures in 2 per cent pepton water, although media containing sugar show wide variations.

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The present study was undertaken to determine the nature of the volatile acids produced by *C. putrefaciens* and to ascertain the limits of variations for different strains at various ages. Twenty-three strains of *C. putrefaciens* were studied in the phosphate buffered pork medium already described. Incubation was at 20°C. without anaerobiosis.

The identification of volatile acids in the complex mixtures obtained as a result of bacterial decomposition presents an extremely difficult problem to the worker in bacteriological science. It appears pertinent to review somewhat in detail the methods heretofore developed and to describe the procedures which we have found useful for this purpose.

The method of Duclaux (1900) has been widely used in volatile acid investigations. Upson, Plum and Schott (1917) have shown that this method as commonly applied is far from exact. Lamb (1917) has demonstrated that the failure of these investigators to secure uniform results was probably due to irregularities in heating and to impurities in the acids employed. Gillespie and Walters (1917) report a comprehensive study of the Duclaux procedure and describe graphic and algebraic methods for determining the mixtures of acids required to give the observed titration sequence. They also describe an electrically heated distilling apparatus which gives very uniform heating. The limitations of the method are enumerated and certain criteria are set up for judging the validity of the results. They point out that Dyer's (1916) steam distillation method has probably as serious objections as the Duclaux procedure.

Phelps and Palmer (1917) separated butyric acid from acetic and formic by means of the solubilities of the quinine salts. We have not been successful in using this method with the acid mixtures obtained from biological material. Orla-Jensen (1904) identified acids qualitatively by the fractional precipitation of their silver salts. Wolf and Telfer (1917) effected a partial separation of the higher from the lower acids by steam fractionation, but their procedure has been found inadequate by Bushnell (1922). It has further been noted by the former investigators that Dyer's color tests are not satisfactory in mixed acids. This

obviously necessitates the separation of the individual acids in the pure state for a reliable identification. Wagner, Dozier and Meyer have divided the Duclaux distillate into two fractions and assumed that all the acetic acid was present in the last fraction and all the valeric in the first fraction, while butyric was assumed to be present in both fractions. Such an assumption is not justified as can easily be seen from the Duclaux tables. As a further test of the reliability of these results they have estimated the weights of the sodium salts which should be obtained on the basis of the calculated acids and compared these with the weights actually obtained. We have satisfied ourselves that such a comparison is usually of no value where three acids are concerned and of questionable significance in deciding whether two acids or three are present. This fact was first noted while studying a distillation sequence for volatile acids from an unknown mixture in which the highest acid was known to be isovaleric and the lowest acetic.

No differences were found between the observed and calculated sequences when either butyric or propionic was assumed to be the intermediate acid. The solutions were obtained by Gillespie's graphic method for three acids, and agreed almost exactly with the observed values. The mixtures were 26.5 acetic, 51.5 butyric, 22.0 valeric, and 7.0 acetic, 55.0 propionic, 38.0 per cent valeric respectively. To determine the validity of using the identity of the observed and calculated weights of the sodium or barium salts as a criterion of the correctness of one of these solutions the mean molecular weights of the sodium salts for the two solutions mentioned were calculated as follows:

$$82 \times 0.265 = 21.7$$
$$110 \times 0.515 = 56.7$$
$$124 \times 0.220 = 27.3$$
$$\overline{105.7} \quad \text{Mean mol. wt. No. 1}$$

$$82 \times 0.070 = 5.7$$
$$96 \times 0.550 = 52.8$$
$$124 \times 0.380 = 47.1$$
$$\overline{105.6} \quad \text{Mean mol. wt. No. 2}$$

It is quite evident that in this particular case any correspondence between the calculated and observed values for the weights of sodium salts would not prove the validity of either assumption. Similar calculations on various other mixtures satisfying a given set of distillation figures have convinced us that usually the checking of results by the weights of the salt obtained is misleading.

The distilling apparatus described by Gillespie (1917) has been found very satisfactory. Using it in conjunction with various

TABLE 1

The Duclaux constants for the apparatus employed

DISTILLATE	PER CENT ACID IN DISTILLATE			
	Acetic	Propionic	Butyric	Iso-valeric
cc.				
10	7.9	11.7	16.3	25.5
20	16.0	22.9	31.7	46.1
30	24.6	34.0	45.1	62.5
40	33.1	44.7	57.4	75.2
50	42.3	55.0	68.4	84.8
60	51.9	65.2	77.7	91.3
70	62.2	74.8	85.6	95.4
80	73.4	83.8	91.9	98.2
90	85.7	92.4	96.8	99.5
100	100.0	100.0	100.0	100.0
Per cent total distilled......	79.0	92.5	97.0	100.0

fractionation procedures, we have been able to separate the highest and lowest molecular weight acids from mixtures of volatile acids from bacteriological material. Knowing the identity of the extreme acids in a mixture and the distillation figures of the original, it is possible to judge with a reasonable degree of certainty whether considerable quantities of a third acid are present.

The constants for the Duclaux apparatus used in this study are given in table 1. The purest acids obtainable were purchased and purified. The acetic and propionic acids were purified by repeated crystallization of the barium salts.

The butyric and valeric acids were freed from higher and lower acids by fractional precipitation of the silver salts and subsequently recovered by steam distillation. Since the Duclaux figures were to be obtained on the acidified sodium salts, a standard procedure was adopted in which a suitable quantity of the sodium salt was treated with sufficient sulfuric acid to liberate the volatile acid and to give an excess of 0.5 cc. of normal acid. The quantities used were 20 and 50 cc. of N/10 volatile acid in a volume of 110 cc. No difference was observed in the constants for these two dilutions. No corrections have been made for

TABLE 2

Duclaux titration of C. putrefaciens

DISTILLED	PER CENT DISTILLATE	
	Obtained	Calculated
cc.		
10	10.8	10.9
20	20.8	21.1
30	30.6	31.0
40	40.3	40 3
50	49.3	49.6
60	58 8	58.5
70	68.1	67.8
80	77.9	77.6
90	88 2	88.0
100	100.0	100.0

absorption of carbon dioxide, since the errors from this source are compensated by similar errors in the unknown.

The volatile acids were steam distilled from the meat cultures and titrated. After the addition of a slight excess of alkali the distillate was evaporated, if necessary. A sample containing 20 to 50 cc. of N/10 mixed acid was acidified with sulfuric acid and used for Duclaux distillation.

A typical Duclaux titration sequence for the volatile acids produced by *C. putrefaciens* in meat medium is given in table 2. Acetic and valeric acids only were assumed to be present and the calculations were made according to Gillespie's algebraic method, using the constants in table 1. The titration sequence corre-

sponded to 83 per cent acetic and 17 per cent iso-valeric. These percentages, of course, refer to cubic centimeters of N/10 acid per 100 cc. of Duclaux distillate and mean that acetic and valeric acid may be present in the molar ratio of 83:17. The ratio of the acids in the original sample is obviously different from that of the distillate. The former can be calculated readily by means of the per cent of total acid distilled as given in the last line of table 1. For this particular case the ratio in the original was 86:14. In the last column of table 2 we have calculated a titration sequence for a ratio of 83 acetic to 17 valeric, showing a fair agreement.

To establish definitely the presence of acetic and valeric acids in the cultures, a combination of the steam distillates from various cultures was made and from this material the pure acids were isolated. This separation was effected at first entirely by steam fractionation; partial precipitation of the silver salts combined with steam fractionation was subsequently developed resulting in a great saving of time.

A sample containing the equivalent of 1035 cc. N/10 mixed acid was steam fractionated according to scheme 1 in which the higher volatile acid fractions appear at the left and the lower at the right.

1035 cc. N/10 acid

200 cc.   400 cc.   419 cc
A   B   C

102 cc.   95 cc.   120 cc.   275 cc.
A_1 A_2 B_1 B_2

51 cc.   48 cc.   694 cc.
A_3 A_4 F_1

20 cc.   28 cc.   210 cc.   433 cc.
A_5 A_6 F_2 F_3
1   1
Valeric acid   Acetic acid
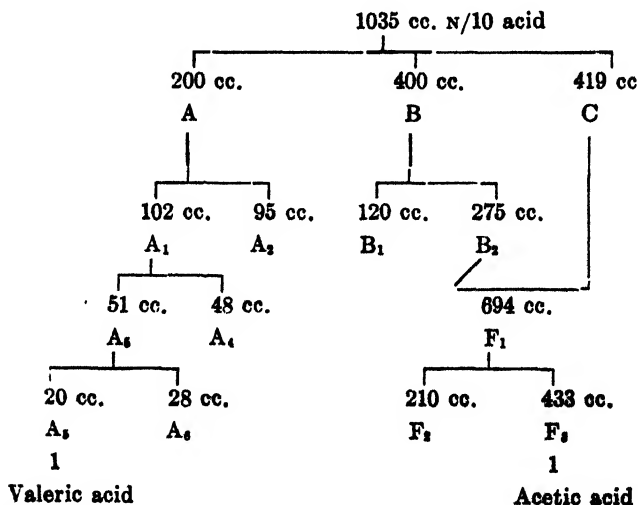
SCHEME 1. STEAM FRACTIONATION OF MIXED VOLATILE ACIDS

The progress of fractionation was followed by Duclaux distillations. Table 3 contains the titration sequences for fractions A_5, B_1, and F_3 the high intermediate, and low molecular weight volatile acids respectively.

It is evident that an excellent separation of acetic acid has been effected. The ease with which 40 per cent of the mixed acid was separated as practically pure acetic acid precludes the presence of more than traces of propionic acid. Since no evi-

TABLE 3

Duclaux titrations on various fractions

DISTILLED	PER CENT OF DISTILLATE							
	Fraction A_5		Fraction B_1		Fraction F_3		Fraction V	
	Found	Calculated	Found	Calculated	Found	Calculated	Found	Calculated
cc.								
10	24.2	24.0	14.5	14 4	8.1	8.2	25.3	25.5
20	44.2	43.7	27.3	27.4	16.4	16.5	45.9	46.1
30	60.6	59 6	39.3	39.2	25.0	25.2	62.2	62.5
40	72.6	72.2	49.8	49.7	33.9	33.8	74.8	75.2
50	82.1	82.0	59.4	59.7	43.1	43.1	84.2	84.8
60	88.7	88.9	68.3	68.2	52.8	52.7	90.8	91.3
70	93.7	93.7	76.4	76.4	62.9	62.9	95.1	95.4
80	96.8	97.0	84.2	84.2	73.9	73.9	97.8	98.2
90	99.0	99.0	92.0	92.0	86.1	86.1	99.2	99.5
Per cent acid estimated in distillate	Butyric 17 Valeric 83		Acetic 50 Butyric 25 Valeric 25		Acetic 97 Butyric 3		Valeric 100	

dence of formic acid was obtained by chemical test there was no point in purifying the acetic acid further.

The intermediate acid fraction B_1 which should contain a relatively high percentage of butyric acid along with valeric and acetic, gives a titration sequence which is exactly satisfied by a mixture of 25 per cent butyric, 25 per cent valeric and 50 per cent acetic. Valeric and acetic acid alone do not give an acceptable solution for this fraction. It should perhaps be noted that the solution for three acids was made by Gillespie's graphic method.

It was desirable to obtain a complete separation of valeric

acid to render the results entirely conclusive. To this end a culture sample equivalent to 400 cc. of N/10 mixed acids was steam-distilled. The first portion of distillate equivalent to 100 cc. of N/10 acid was collected in caustic soda and 4 cc. of normal silver nitrate added. The precipitated silver salt was filtered, washed and the salt transferred to the steam distilling apparatus, acidified and distilled. The distillate equivalent to 30 cc. of N/10 acid was subjected to a Duclaux determination with the results reported under fraction V in table 3. The close correspondence between the observed constants and the constants for pure valeric acid is evident. Fraction V was subsequently

TABLE 4

Volatile acids produced by C. putrefaciens of different ages

	C. PUTREFACIENS 2						C. PUTREFACIENS 6					
	Tube (1)			Tube (2)			Tube (1)			Tube (2)		
AGE	N/10 acid per 100 grams	Acetic	Valeric	N/10 acid per 100 grams	Acetic	Valeric	N/10 acid per 100 grams	Acetic	Valeric	N/10 acid per 100 grams	Acetic	Valeric
days	cc.	per cent	per cent	cc.	per cent	per cent	cc.	per cent	per cent	cc.	per cent	per cent
4	140	88.5	11.5	104	88.5	11.5	148	90.0	10.0	156	89.5	10.5
6	228	88.5	11.5	228	87.5	12.5	240	90.0	10.0	244	88.5	11.5
10	352	88.0	12.0				348	87.0	13.0	356	86.0	14.0
16	388	87.5	12.5	429	86.0	14.0	432	87.0	13.0	394	86.5	13.5
35	552	85.0	15.0	500	86.0	14.0	516	86.0	14.0	524	86.0	14.0

steam-fractionated into two portions, each equivalent to 15 cc. N/10 acid. The Duclaux constants were identical within the limits of errors, furnishing further proof of the purity of this sample. The data just presented indicate that *C. putrefaciens* produces three acids, acetic, butyric, and isovaleric, the acetic constituting more than three quarters of the total.

It is a mathematical fact that the Duclaux method, even when carried out with meticulous care, gives results which are uncertain with such a combination of acids as we have to deal with here. This is because unavoidable experimental errors are unduly exaggerated by the necessary computations. It is therefore essential either to ignore the presence of one of the higher

acids, preferably the intermediate, or to run a sufficiently large number of check titrations to insure a significant average. For calculating the acids in individual cultures we have assumed that valeric and acetic only are present. To obtain an estimate of the three acids the titration sequences for a large number of individual cultures seven to twelve days of age were averaged and the resultant was solved graphically.

It was desirable to determine the limits of variation in volatile acid production for *C. putrefaciens* over a wide range of age.

TABLE 5

Comparison of Duclaux titrations for maximum and minimum acetic acid production

DISTILLED	MAXIMUM		MINIMUM	
	Per cent distillate observed	Per cent distillate calculated	Per cent distillate observed	Per cent distillate calculated
cc				
10	10.1	10.1	10.9	11.1
20	19.8	19.8	21.3	21.4
30	29 4	29.4	31.1	31.5
40	38.6	38.4	40 6	40.7
50	47.6	47.7	49.9	49.9
60	57.0	56.9	59.1	59 0
70	66 6	66.4	68.4	68 2
80	76.5	76.6	78.0	77.9
90	87.3	87.4	88.4	88.2

Note: Maximum corresponded to 87.5 acetic; 12.5 valeric in Duclaux distillate which is equivalent to 90:10 in the culture. Similar figures in the minimum are 82:18 for distillate and 85:15 in culture.

In table 4 data are tabulated on two strains of *C. putrefaciens* for several different ages. Duplicate tubes were analyzed at each age. The values obtained refer to the mole per cent of acetic and valeric acids in the culture.

The results suggest that a slight increase in the relative amount of the higher volatile acids occurs with increasing age.

The titration sequences for maximum and minimum acetic acid production which are specifically those for *C. putrefaciens* 6, tube 1, for four days and *C. putrefaciens* 2, tube 1, for thirty-five days (table 4) are given in table 5.

It appears from table 5 that even over a wide range of ages the titration sequences lie within a narrow zone. The close correspondence between the observed and calculated values for both titrations, assuming valeric and acetic acid only, indicate the difficulty of solving for the three acids.

TABLE 6

Volatile acids produced by different strains of C. putrefaciens in meat medium

STRAIN NUMBER	AGE	N/10 V. A. PER 100 GRAMS	PERCENTAGE	
			Acetic	Valeric
	days	*cc.*		
1	7	321	85.0	15.0
2	7	356	86.0	14.0
3	7	320	86.0	14.0
4	7	330	87.5	12.5
5	7	335	86.5	13.5
6	7	348	87.0	13.0
7	8	324	87.5	12.5
8	8	372	86.5	13.5
9	8	352	87.0	13.0
10	8	327	86.5	13.5
11	7	296	85.5	14.5
12	7	294	86.0	14.0
13	7	329	87.5	12.5
14	7	276	88.0	12.0
15	7	300	88.0	12.0
16	7	300	88.0	12.0
17	8		88.0	12.0
18	10	364	85.5	14.5
19	10	373	85.0	15.0
20	10	386	85.0	15.0
21	11	372	86.5	13.5
22	11	376	85.5	14.5
23	11	396	86.0	14.0
24	11	392	85.5	14.5

In table 6 the volatile acids produced in meat medium by all available strains of *C. putrefaciens* are compared at ages of seven to twelve days. This table demonstrates that little variation exists in the proportions of the volatile acids produced by a large number of strains of *C. putrefaciens*.

We have previously demonstrated that some butyric acid is

present in cultures of this anaerobe. It is of interest to attempt a quantitative estimate of this acid. This can best be done, for reasons already noted, by averaging a large number of Duclaux titrations. The second column of table 7 represents the average titration sequence of the twenty-four cultures summarized in table 6. The observed average was solved graphically for acetic,

TABLE 7

The proportions of acetic, butyric and valeric acids produced by C. putrefaciens

DISTILLATE	PER CENT OF DISTILLATE		
	Observed average	Calculated for acetic, butyric, valeric	Calculated for acetic and valeric
cc.			
10	10.4	10.4	10.6
20	20.5	20.3	20.7
30	30.0	30.4	31.2
40	39.5	39.4	39.6
50	48.8	48.8	48.8
60	58.1	58.0	58.0
70	67.5	67.5	67.3
80	77.4	77.4	77.2
90	87.9	88.0	87.8

TABLE 8

Volatile acid production by C. putrefaciens in various media

CULTURE	MEDIUM	AGE	ACETIC ACID	VALERIC ACID
		days	*per cent*	*per cent*
C. putrefaciens 6....................	Gelatin	16	91.0	9.0
C. putrefaciens 5................ ...	Gelatin	24	92.5	7.5
C. putrefaciens 6..............	Pork heart	30	81.5	18.5
Average all strains...............	Meat	7–11	84 5	15.5

butyric and valeric acids, giving 82, 7 and 11 mole per cent respectively in the distillate. This corresponds to 85, 6 and 9 mole per cent of these acids in the original culture. In column three we have computed the theoretical titration sequence for this graphic solution. If acetic and valeric acids only are assumed present the solution is 84.5 acetic and 15.5 valeric in the dis-

tillate corresponding to 87.5 and 12.5 mole per cent in the original. In the last column the calculated titration for these values is recorded. While the figures reported in table 7 show a close agreement, a definite trend can be noted in the two acid calculation which is not evident when the calculation is made for three acids. This trend is more noticeable during the algebraic solution for these two acids, and is caused by the presence of some butyric acid. Since the calculations depend on deviations of a few tenths only, it is obvious that no great significance attaches to a solution for three acids based on a single titration sequence, and explains why we have presented the results for individual

TABLE 9

Duclaux titrations of various anaerobes in meat medium.   Age ten days

ORGANISM	PER CENT DISTILLATE IN								
	10 cc.	20 cc.	30 cc.	40 cc.	50 cc.	60 cc.	70 cc.	80 cc.	90 cc.
C. putrefaciens Av............ ...	10.4	20.5	30.0	39.5	48.8	58.1	67 5	77.4	87.9
C. sporogenes.....	15.0	28.0	39.6	49 9	59.2	67.7	75.5	83.3	91.2
C. flabelliferum	14.6	28.2	40.3	51.1	60.9	69.6	78.0	86.0	93.6
C. Reading..	16.0	29.5	41.6	52.1	61.9	70.4	78.0	85.2	92.3
C. parasporogenes	15.0	28.2	40 2	50.7	60.4	68.8	76.9	84.4	92.0
C. bifermentans.... ... .·...	14.9	28.0	39.8	50.4	59.6	68.4	76.3	84.0	91.6
C. putrificum..........	12.0	22.7	33.4	43.4	53.2	62.6	71.7	80.8	90.0
C. botulinum Type A............... ..	16.8	31.1	43.8	55.0	64.6	73.2	80.6	87.4	93.6
C. botulinum Type B.......	16.2	30 2	42.7	53.5	63.0	71.4	79.0	86.2	93.0

cultures in terms of two acids only.   It is believed that the molecular ratio 85:6:9 for acetic, butyric and valeric based as it is on a large number of observations of different strains is far more significant than any single observation could ever be.

Limited data on the volatile acid production in gelatin and pork heart medium are available.   These are presented in table 8. Slight quantitative differences appear to exist between the different media, however, the predominance of acetic acid in all of them is noteworthy.

A comparison of the titration sequences of *C. putrefaciens* with those of the common anaerobes is illuminating and demonstrates that the former differs markedly from all the other anaerobes

investigated. In table 9 the titration sequences for the various anaerobes in meat medium at ten days age are compared with the average titration sequence of *C. putrefaciens*. The analyses for the other anaerobes were run on triplicate tubes, the values reported being the mean of three closely agreeing Duclaux distillations. No attempt has been made to identify the individual acids in the various cultures, but it is evident that *C. putrefaciens* produces relatively more acetic acid than any of the others. *C. putrificum* seems to approach the distillation figures for *C. putrefaciens* rather closely, however the former is a slow growing organism and at thirty to thirty-five days the titration sequences are practically identical with the other anaerobes.

It is to be regretted that no culture of *C. tetani* was available for comparison. Wagner, Dozier, and Meyer (1924) have reported that this organism produces a preponderance of acetic acid and that in one instance they have identified the volatile acid as practically pure acetic acid.

DISCUSSION

The extensive study of the volatile acid production by *C. putrefaciens* reported here permits the conclusion that there is but slight variation in the Duclaux titration sequences with time. In table 4 there is evidence of a slight increase in the higher molecular weight volatile acids amounting at most to 5 per cent computed as valeric acid. Furthermore, twenty-four strains are shown to give very consistent results at ages of from seven to twelve days, showing that the proportion of volatile acids produced by this organism is a constant property for all strains. Wagner, Dozier and Meyer (1924) (1925) report that cultures of *C. sporogenes* and *C. botulinum* contain relatively more higher molecular weight volatile acids the older the culture, while their results on *C. tetani* suggest that different cultures of this organism may produce widely different proportions of volatile acids. In contrast to these results our data indicate that a remarkable constancy exists in the proportions of the volatile acids produced in meat medium by all strains of *C. putrefaciens* at all ages. It is evident also that the Duclaux titration is sufficient to differ-

entiate this organism from most of the common putrefactive anaerobes in meat medium.

The writers feel that Gillespie's distilling apparatus coupled with the methods he has outlined for solving the distillation data are much more accurate than the methods ordinarily used. The trustworthiness of the data is greatly increased if distilling constants are determined for the individual apparatus. We believe that the ordinary method of comparing distillation data with Duclaux tables leads to considerable errors in the conclusions. For example the data on *C. botulinum* in meat medium as reported by Wagner, Dozier and Meyer (1925), if calculated according to Gillespie's method using Duclaux's constants, give values of 23, 38 and 39 per cent of acetic, butyric and valeric (molar ratio approximately 1:2:2) as contrasted to the reported values of 16.5, 58.5 and 25 per cent (molar ratio 2:7:3). Needless to say the values calculated from the former figures are much closer to the observed than those given by these investigators.

Waksman and Lomanitz (1925) concluded that the mechanism of protein and amino acid decomposition varied with the type of organism. With fungi, they report smaller accumulation of ammonia the higher the carbon content of the amino acid. No data are presented, however, concerning the fate of the carbon residues. Apparently they believe that the carbon residues are partially synthesized into bacterial protoplasm and partially oxidized to carbon dioxide as an energy source.

We have shown in the second paper of this series that in the case of *C. putrefaciens* a molecular equivalence exists between volatile acid and ammonia. This paper demonstrates that the volatile acids produced do not greatly vary either with age or strain. It has also been noted that nonvolatile acid is not produced and that carbon dioxide is formed in the proportion of 1 mole to 3 of ammonia. It is impossible to fix the origin of carbon dioxide. It may possibly arise from a beta oxidation of the volatile acids. The fact that there is a slight accumulation of higher volatile acid in the older cultures seems to render such a conclusion improbable. There appears to be no reason why the carbon dioxide may not be formed simultaneously with the

deamination process. Since the carbon residues from deamination exist in the form of volatile acids in the exact proportion to neutralize the ammonia, one must either conclude that only a portion of the molecule is utilized in the synthesis of protoplasm or that amounts so small that they are beyond the limits of the methods are involved.

Wagner, Dozier and Meyer (1925, p. 401) evidently look upon lactic acid as a connecting link between the protein and carbohydrate metabolism. We have failed to find evidence of non-volatile acid production and, therefore, it seems doubtful that such a connection exists in the case of this organism.

SUMMARY

1. The volatile acids produced by *C. putrefaciens* are identified as acetic, butyric and valeric.

2. A remarkable constancy has been demonstrated for the Duclaux titrations of various strains at various ages.

3. The average proportions of the various acids in mole per cent are acetic 85, butyric 6, valeric 9.

4. A comparison has been made of the titration sequences of *C. putrefaciens* and the common anaerobes, which demonstrates that the former differs markedly from the latter.

REFERENCES

BUSHNELL, L. D. 1922 Jour. Bacteriol., 7, 373.
DUCLAUX, E. 1900 Traité de Microbiologie, 3, 385.
DYER, D. C. 1916 Jour. Biol. Chem., 28, 445.
GILLESPIE AND WALTERS 1917 Jour. Amer. Chem. Soc., 39, 2027.
LAMB, A. R. 1917 Jour. Amer. Chem. Soc., 39, 746.
ORLA-JENSEN 1904 Landwirtschaftliches Jahrbuch der Schweiz, p. 309.
PHELPS AND PALMER 1917 Jour. Biol. Chem., 29, 199.
SELIBAR, C. 1914 Rev. Gen. Bot., 25, 589.
UPSON, PLUM AND SCHOTT 1917 Jour. Amer. Chem. Soc., 39, 731.
WAGNER, DOZIER AND MEYER 1924 Jour. Infect. Dis., 34, 63.
WAGNER, DOZIER AND MEYER 1925 Jour. Bacteriol., 10, 321.
WAKSMAN AND LOMANITZ 1925 Jour. Agric. Research, 30, 263.
WOLF AND TELFER 1917 Biochem. Jour., 11, 213.

FILTERABLE VIRUSES

A CRITICAL REVIEW[1]

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In all fields of work, times come when one must stop and take thought. New facts, new ideas, and new suggestions alter lines of endeavor in every field of research. We are here today for the purpose of taking thought concerning the knowledge of the so-called filterable viruses and the diseases caused by them. I have been asked to give a critique of this knowledge. It is quite obvious that I shall be unable within an hour to analyze thoroughly and to criticize authoritatively all the work in this field. Therefore, I shall review quickly some facts and ideas concerning this group of diseases as a whole and then discuss a few reports concerning several of its individual members.

In table 1 are listed most of the diseases which are included by different observers in the group under discussion. The etiological agents concerned in these diseases, or groups of them, have been given a variety of names, e.g., filterable viruses, invisible microbes, ultra-microscopic viruses, inframicrobes, protista, microplasms, chlamydozoa, and strongyloplasms. A superficial examination alone is convincing that none of these names is applicable to all of the etiological agents. Names, however, facilitate the interchange of facts and ideas between individuals. For practical purposes, then, the term "filterable viruses," mainly because of its wide usage, is as satisfactory as any name suggested. Throughout this discussion the term "filterable viruses" will be employed in a noncommittal way to designate certain active

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[1] Read before the Society of American Bacteriologists, December 29, 1926.

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TABLE 1

Majority of the diseases which have been placed in the filterable virus group by different workers

Bacteriophage

Mosaic diseases of plants (infectious chlorosis)

Sacbrood
Wilt of European nun moth
Wilt of gypsy moth caterpillar
Jaundice of silk worms

Episoötic of guinea pigs
Hog cholera
Cattle plague (Rinderpest)
Pernicious anemia of horses
Virus III infection of rabbits
Foot-and-mouth disease
    1. Type A
    2. Type O
Vesicular stomatitis of horses
Paravaccinia (No report on filtration)

Swine-pox
Goat-pox
Sheep-pox
Horse-pox
Cow-pox
Smallpox
Varioloid
Alastrim
Chicken-pox   No reports
Herpes zoster   on filtration
Symptomatic herpes
Encephalitis
    1. Lethargic
    2. Following vaccination
    3. Japanese 1924
    4. Koritschoner
    5. Australian X disease
Poliomyelitis
Rabies
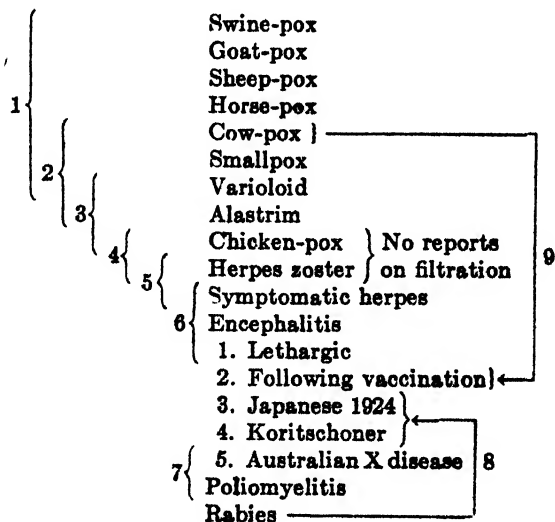
Borna's disease
Fowl plague and plague of blackbirds
Guinea-pig paralysis
Distemper of dogs

TABLE 1—*Continued*

Trachoma and inclusion blenorrhea
Infectious papular stomatitis of cattle
Molluscum contagiosum
Warts
Contagious epithelioma (fowl-pox)
  1. Chickens
  2. Pigeons
Infectious myxomatosis of rabbits
Rous sarcoma of chickens
Leukemia of chickens
Lymphocystic disease of fish ⎫
Epithelioma of fish ⎬ No reports on filtration
Carp-pox ⎭

Mumps(According to Kermorgant, a spirochetal disease)
Agalactia(According to Bridré, a bacterial disease)
Salivary gland disease of guinea pigs

Measles(rubeola)
German measles (rubella)(No report on filtration)

Grippe (influenza)(According to Olitsky and Gates a bacterial disease)
Common colds

A Insect-borne	A	Nairobi disease of sheep
		Catarrhal fever (blue tongue) of sheep
		African horse sickness
		Pappataci fever
		Dengue fever
		Yellow fever (According to Noguchi a spirochetal disease)
	B Rickettsia diseases	Typhus fever
		Trench fever
		Rocky Mountain spotted fever
		Heartwater disease
		Flood fever of Japan
B Bacterial diseases		Orroya fever and verruga peruviana
		Pleuropneumonia
		Avian diphtheria
		Scarlet fever

transmissible agents which are capable of producing pathological conditions in bacteria, plants, insects, fish, birds, and mammals, and which by general consent are more or less limited for the moment to the etiological agents of the diseases listed in the table.

The arrangement of the diseases in the table is for convenience of discussion and carries no taxonomic significance. In the first place, filterability of the etiological agents does not sharply delimit this group of diseases, as it is well known that the viruses share this characteristic with certain small bacteria and vibrios, and also with some spirochetes and protozoa. Furthermore, in regard to the etiological agents of some of the diseases wtihin the group, e.g., chicken-pox and paravaccinia, no filtration experiments have been recorded. The viruses of still other diseases within the group, e.g., typhus fever, and vaccinia, are either not filterable or filterable with the greatest difficulty. In the past all attempts to classify these diseases have been unsuccessful and there is every reason to believe that such attempts are still premature.

The diseases listed do not form a homogeneous group and some of them should be omitted. The evidence that epizoötic of guinea pigs is a virus disease is not convincing. The Rickettsia diseases do not belong here' and there is considerable doubt as to how long the other insect-borne diseases will remain on the list. This is particularly true of yellow fever. Most observers no longer consider scarlet fever a virus disease. M'Fadyean, as early as 1908, suggested that pleuropneumonia be classified with bacterial diseases. Oroya fever and fowl diphtheria, in view of Noguchi's work and Bordet's experiments respectively, do not belong here. Enough has been said to convince one of the heterogeneity of the diseases listed in the table. In fact they exhibit so many differences that a discussion of the filterable viruses almost amounts to a separate discussion of each disease. Such a state of affairs is due to the fact that the filterable virus group has been used to a considerable extent for the indiscriminate segregation of infectious diseases of unknown etiology. Therefore, it is not unlikely that some of them will be shown to be caused by small bacteria or protozoa. When this occurs, such

diseases should be removed from the filterable virus group and given their correct position in the classification of diseases.

EPIDEMIOLOGY

From the epidemiological standpoint the group of filterable virus diseases is notable chiefly because of the remarkable differences exhibited by its members when compared one with another. Some are extremely contagious, e.g., smallpox; others, although inoculable, are not spread by ordinary contact, e.g., rabies; others are insect-borne, e.g., dengue fever and yellows of asters; and still others are transmitted only by grafting, e.g., certain infectious chloroses of plants (Baur). In general, however, the epidemiological problems presented by the virus diseases in regard to regional distribution, seasonal variation, host susceptibility, and virulence are similar to the problems found in connection with other infections.

IMMUNITY

Since immunity, either natural or acquired, plays such an important rôle in all epidemiological studies, a discussion of it at this point is not out of place. With a few exceptions, diseases produced by the filterable viruses, if recovered from, lead to a lasting immunity. In this respect virus diseases differ from those caused by ordinary bacteria. This is not universally true, however, since one attack of typhoid fever produces in the recovered individual a fairly lasting immunity. Many questions have arisen in regard to this kind of immunity, but so far they have not been satisfactorily answered. Nevertheless, a few of the possibilities will be discussed.

Adults, forty years of age, who had measles or chicken-pox when 1 year old are still refractory to reinfection. Some investigators state that the serum of these refractory individuals is slightly protective even after a period of 39 years, or contend that the cells are still supplied with sessile antibodies or an increased ability to make them. The majority of the cells in an adult, however, are not the same cells possessed by that individual when

1 year of age.   If it is a question of sessile antibodies, then one must suppose that these characteristics are passed on to daughter cells.   On the other hand, a child born of immune parents becomes susceptible to measles and chicken-pox within a few months after birth.   Is it possible then that the lasting immunity is due to repeated infections so mild that they attract no attention except in the first instance?   In regard to diseases as prevalent as measles and chicken-pox this might serve as an explanation. On the other hand, one can hardly explain the persistent protection against poliomyelitis and smallpox upon such grounds.

Another possible explanation for the lasting immunity is that it is due to a prolonged sojourn of the virus in the body or perhaps to its persistence in an individual once infected.   Winkler, in his review of immunity to vaccine virus, suggests this possibility. When confronted with the idea one invariably says that it is impossible, because, if it were true, every one would spread measles and chicken-pox.   This would not necessarily be the case, however.   Typhoid bacilli have been found in the walls of gall-bladders many years after attacks of typhoid fever.   Furthermore, when the mucous membranes lining the gall-bladders are normal, there is little danger of the disease being spread by these carriers.   Most human beings carry tubercle bacilli, but only a few spread tuberculosis.   Individuals harboring *Treponema pallidum* are not always infectious, particularly in the latent stages of syphilis.   Furthermore, syphilis is an excellent example of a disease in which there is a persistent infection coincident with a refractory state in the host to reinfection.   For information concerning discussions of this paradox one should read Chesney's review, "Immunity in Syphilis" (Medicine, 1926, **5**, 463).

Now, in regard to virus diseases, is there any evidence (1) of a prolonged or persistent infection, (2) of a coexistence of infection and refractory state in the host to reinfection from without, and (3) of a causal relation between the prolonged or persistent infection and the lasting immunity?   There is considerable evidence that a prolonged infection occurs in some virus diseases and also that this infection can persist for a long time in a host refractory to reinfection.   De Kock found a horse's blood infectious seven

years after an attack of pernicious anemia. Sir Arnold Theiler speaks of the persistent infectious nature of the blood of horses that have recovered from pernicious anemia. He also states that the blood of horses inoculated with African horse sickness may remain infective for periods up to three months after inoculation. In the majority of instances, a plant once infected with mosaic virus is never free from it. Vaccine virus has been recovered from the lymph nodes of an animal 28 days after inoculation and twenty-two days after the animal's skin was refractory to reinfection. A prolonged infection occurs in contagious epithelioma, and in regard to this disease Lipschütz says, "Der immune Organismus ist Parasitenträger." Furthermore, Cole and Kuttner have shown that the "salivary gland virus" of guinea pigs can be obtained at will from immune animals, and in this particular instance it appears that a pig once infected continues to carry the virus indefinitely in spite of a refractory state to reinfection from without. The question as to whether the lasting immunity is dependent *per se* upon a prolonged sojourn or persistence of viruses in the body cannot be answered at the present time. Nevertheless, such an idea, novel in regard to virus diseases, is worthy of serious consideration.

Another interesting feature concerning the immunity to virus diseases is the fact that only active virus protects against a second inoculation of the same virus. In other words it is doubtful, with a few exceptions, whether injection of a virus completely inactivated leads to a protection against the same virus in an active state. Furthermore, virucidal properties do not appear in the serum of naturally resistant animals which have received repeated injections of active virus. Therefore, it seems that an actively acquired immunity and evidences in the serum of such an immunity are dependent in some way upon an actual infection with the virus, even though it be so mild at times as to give rise to no symptoms.

The degree of active immunity usually exhibited by individuals recovered from virus diseases seems disproportionate to the amount of passive protection afforded by their sera. This fact has led many observers to believe that the protection against

virus diseases is predominantly a tissue immunity rather than a humoral one.   Be that as it may, protective substances do occur in the sera of individuals who have recovered from certain virus diseases.   This is particularly true when animals have been hyperimmunized by means of repeated injections of the viruses, as is evidenced by Gordon's work concerning immunity to vaccinia.

Virucidal properties, precipitins, and complement-fixing antibodies have been demonstrated in the sera of individuals who have recovered from certain of the virus diseases.   Gordon and others believe that agglutinins also exist in the sera of animals that have recovered from vaccinia, inasmuch as "vaccine granules" are agglutinated by such sera.   Until these granules have been definitely shown to represent only vaccine virus, one must look upon the supposed agglutinins with a great deal of suspicion.

PREVENTION OF DISEASE CAUSED BY VIRUSES

The prevention of diseases caused by viruses depends upon the protection of susceptibles from exposure or upon their immunization.   Quarantine measures are established for protection but frequently they are ineffectual.   The eradication of insects or protection against them controls the spread of insect-borne diseases.   Furthermore, susceptible species can be replaced by naturally insusceptible ones, and at times it is possible to breed refractory hybrids.   This method has been used advantageously where animals and plants are concerned.   Unfortunately, however, such methods cannot be employed in dealing with all animal diseases, nor can they ever be used in dealing with human diseases.   Under these conditions attempts are made to decrease the number of susceptibles by means of vaccination with attenuated or modified viruses.

FILTERABILITY

Since the discovery of the first filterable virus in 1892, it has been determined by means of different kinds of filters that many diseases are caused by active agents smaller than ordinary

bacteria. Some are presumably much smaller and are most likely optically immeasurable. Others, however, do not seem to be so small and concerning the filterability of these there is much discussion. Methods of filtration are crude and inaccurate and the most one can say regarding the viruses is that under given experimental conditions they either pass or do not pass through certain filters. The failure to pass through a filter, however, is certainly not determined in every instance by the size of the virus. The electrical charge on the virus, the electrical charge on the filter, the adsorption of the virus by aggregates of protein or by cell detritus, the amount of protein or other substances in the virus emulsion, the temperature at which the filtration is conducted, the amount of negative or positive pressure employed, the duration of filtration, and other factors, not mentioned or not known, serve to influence the results of all filtration experiments. Furthermore, sufficient attention has not been given to the possibility that some filters may not only hold back certain viruses but may also inactivate them in some manner so that they can never become active again.

Filters, in spite of their faults, are useful in working with diseases of unknown etiology, and by means of them one is able at times to determine quickly whether a given disease is produced by an agent smaller than ordinary bacteria. Sometimes, however, small bacteria may still contaminate the filtrates or two viruses may be present in the filtered material. Therefore, filtrates from uncontrolled and even from well controlled sources may contain more than one active agent, some of which may be cultivated on simple or on special media. All investigators should be extremely careful in working with filtrates not to be misled by their findings and ascribe to an active agent an etiological rôle in a disease with which it has nothing more than accidental connection.

SIZE

Very little has been recorded in regard to the size of many of the viruses other than that they pass through certain kinds of filters. It is obvious that this method only indicates roughly

that the viruses which pass through tight Chamberland candles are very small. No virus has been obtained in an absoutely pure state. Not even the washed granules of vaccine virus can be accepted as representing only virus. Therefore it is impossible to say that virus alone is being filtered rather than virus attached to aggregates of protein or particles of degraded cells. Nevertheless, attempts have been made in various ways to determine the size of a few viruses. According to d'Herelle, the diameter of the phage is 20 to $30\mu\mu$. Bechhold and Villa state that its diameter is $>35\mu\mu$ and $<200\mu\mu$. Duggar and Karrer believe that the virus of tobacco mosaic is approxmately the same size as the colloidal particles of fresh 1 per cent hemoglobin, $30\mu\mu$.

The size and weight of molecules of crystalline egg albumen and crystalline hemoglobin are not agreed upon. Bechhold states that an aggregate of 50 molecules of egg albumen is >4 and $<10\mu\mu$ in diameter. According to Du Noüy, however, 1 molecule of egg albumen is $4.1\mu\mu$ in diameter. If it is difficult to determine the size of molecules of relatively pure crystalline substances, what hope is there at present of ascertaining the size of the viruses which have not been obtained in a pure state? Furthermore, it is useless to pretend to know what is the lower limit in point of size for living things. In general, however, it can be said that many viruses are probably of sufficient size to exist in a living state, and that others are probably small enough to satisfy the demands of those who insist that they are not possessed of life.

CULTIVATION

Following the discovery of the first filterable virus, thirty-four years ago, numerous workers have claimed to have successfully cultivated *in vitro* by means of simple or complex media one or more of these active agents. Since all claims cannot be discussed, only the outstanding ones will be considered. The term *in vitro* will be avoided as there seems to be no agreement as to its exact meaning. I shall, therefore, discuss the cultivation of viruses in the presence or absence of living cells.

There is no reason to doubt that vaccine virus, herpes virus,

typhus fever virus, the virus of Rous' sarcoma, and the virus of Rocky Mountain spotted fever have been successfully cultivated in the presence of living cells in tissue cultures. Moreover, Levaditi has stated that the virus of poliomyelitis either survived or multiplied in fresh spinal ganglia (monkey) placed in plasma Harde was able to grow vaccine virus in the presence of living corneal cells, but, if the cells were killed by freezing or by hypertonic salt solution, the virus failed to multiply. The virus of fowl plague is cited as one that has been cultivated *in vitro*. One might ask, however, if it has been cultivated in the absence of living cells, inasmuch as a large amount of blood was added to the medium employed. Furthermore, Landsteiner and Berliner found that the virus would not multiply if laked or frozen blood was used, and stated in their report that one could not say that growth of the virus had taken place in a lifeless medium. The statement is frequently seen that Bordet cultivated the etiological agent of contagious epithelioma of chickens. Such statements are incorrect. Bordet claims to have cultivated a small bacterium which causes avian diphtheria. Furthermore, he specifically states that the bacterium does not cause contagious epithelioma and that avian diphtheria and contagious epithelioma are two distinct diseases in spite of the view held by some investigators.

There are excellent reasons for stating that the etiological agents of foot-and-mouth disease, trachoma,[2] rabies, and poliomyelitis have not as yet been cultivated in the absence of living cells. The evidence is insufficient to convince one that "globoid bodies" are etiologically associated with poliomyelitis (Amoss). A consideration of the survival or the multiplication of the virus of poliomyelitis in the presence of pieces of fresh kidney is not germane to the discussion. Streptococci have been given an etiological rôle in many virus diseases. It is unlikely, however, that they cause all of the following diseases: poliomyelitis, lethargic encephalitis, influenza, measles, and German measles.

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[2] Since this paper was submitted for publication Dr. Noguchi has reported his recent work concerning the cultivation of the etiological agent of trachoma.

Gye's reports concerning the cultivation of the virus of Rous' chicken sarcoma will be discussed further on.

Recently Olitsky reported that he was able to cultivate the virus of tobacco mosaic in a simple medium presumably free of cells. Mulvania and Purdy, working independently, were unable to confirm his work and suggested that his results are open to several interpretations. An observation of the kind reported by Olitsky, if correct, is one of a fundamental nature and significance. In fact, it is of almost as much significance as an observation upon the cultivation of bacteriophage in the absence of living bacteria. Unequivocal confirmation of Olitksy's work, therefore, would settle one of the most important problems in the whole field.

In general it can be said that no worker has proved that any of the etiological agents of the diseases in table 1 down to mumps is susceptible to cultivation in the absence of living cells. A satisfactory explanation of the difficulty experienced in cultivating the viruses on artificial media is not easily found. Their small size alone should not make them insusceptible to cultivation. Nor does it seem to be a question of delicacy or sensitiveness, because many of them are extremely resistant to chemical and physical agents. Therefore, the viruses appear to be obligate parasites in the sense that their reproduction is dependent upon living cells. Whether this reproduction occurs intra- or extra-cellularly is a debated question.

CELL TYPES IN RELATION TO VIRUS REPRODUCTION

In view of the fact that viruses apparently multiply only in the presence of living cells, it is advisable to ascertain what kinds of living cells promote their reproduction best, and what effect upon the cells is induced by this reproduction.

Species specificity. A remarkable species specificity is exhibited by many viruses. The Rous sarcoma grows only in chickens. Sanarelli's virus of infectious myxomatosis and Virus III are active only in rabbits. The "salivary gland virus" described by Cole and Kuttner apparently affects only guinea

pigs. A wilt virus that attacks one kind of caterpillar is innocuous for other caterpillars. The virus of poliomyelitis is active only in man and the monkey.

Importance of young cells. Frequently young cells seem essential for the activity of viruses. The bacteriophage multiplies only in the presence of young bacteria. Old or dead bacteria are not lysed except in the presence of young living forms. The activity of mosaic viruses is manifested only in young leaves. This applies also to the infectious chloroses transmitted by grafting only. Virus diseases usually attack insects in certain stages of development. In the higher forms of life virus activity is also best exhibited not in old, undernourished, sickly individuals, but in young healthy ones. Injury also plays an important rôle in the infectiousness of many virus diseases, not the rôle, however, of furnishing a *nidus* of dead tissue for the growth of the viruses, since dead tissues do not promote their growth, but the rôle of furnishing young cells or growth-promoting factors usually found in their vicinity. Injury necessitates repair. Restoration, as is well known, is accompanied by young cells. In a rabbit's cornea and skin the activity of vaccine virus, herpes virus, and Virus III is first seen along the lines of scarification. Furthermore, the evidence of this activity is first found in the young cells filling in the defects caused by the injury. Old cells may become involved later (see fig. 5). Rous and Murphy, working with chicken sarcomas, observed phenomena that seem to be related to injury. Injections of virus emulsion and kieselguhr give rise to more rapidly appearing and more diffusely growing tumors than does the inoculation of virus emulsion alone. More metastases are found in the ovaries during egg laying seasons than during quiescent periods. These phenomena are probably due to the presence of young cells participating in the repair of injured tissues.

Cytotropic phenomena. In some diseases produced by ordinary bacteria and in many caused by viruses, a certain amount of "selective tissue localization" is apparent. Variola, chickenpox, and contagious epithelioma usually attack the skin; rabies and poliomyelitis, the brain; Rous' sarcoma, mesodermal tissue.

Inasmuch as vaccine virus, herpes virus, and chickenpox virus attack both ectodermal and mesodermal cells, it seems that a position of exaggerated importance has frequently been accorded this phenomenon of "selective tissue localization." Nevertheless, some viruses, e.g., the virus of rabies and poliomyelitis, exhibit a remarkable affinity for cells of certain tissues and apparently can neither multiply nor produce signs of disease unless they come into a close relation with these cells.

Injury naturally plays an important part in the infectiousness of diseases that exhibit pronounced cytotropic phenomena. In this case it is not the function, however, of providing young cells, but the rôle of mechanically making it possible for the viruses to come in contact with susceptible types of cells.

EFFECTS OF VIRUSES UPON CELLS

It has been shown that some viruses multiply only in a restricted number of hosts, that frequently this multiplication occurs only when the virus is in close relation with certain types of cells, and that young, actively growing cells play an important rôle in the infectiousness of many virus diseases. Now a word will be said in regard to the effects produced in cells by viruses. At first the involved cells show a remarkable increase in size, often with amitotic division of the nuclei. The increase in size gives one the impression that it is due to growth phenomena and to imbibition of fluid. This process is spoken of as ballooning degeneration. Eventually the cells die and go to pieces. This process is spoken of as colliquation (see figs. 1, 2, 3 and 4). Two forces seem to operate: one stimulating the cell, the other destroying it. Consequently the picture produced by a virus disease is more or less dependent upon which of these forces predominates. Chicken-pox, foot-and-mouth disease, zoster, variola, and lysis of bacteria by phage are diseases in which destructive agencies predominate. Rous' sarcoma, contagious epithelioma, fowl leukemia, and warts are diseases in which stimulating forces are dominant. A few observers have attempted to classify the cytotropic viruses under cytolytic and cytokinetic

headings with subdivisions under each according to the type of cells involved.   Such a classification is premature.   In table 1, however, a suggestion of the possibility is seen in the arrangement of the diseases.

It is not known whether the viruses multiply intra- or extra-cellularly.   Nevertheless, they have a profound influence upon cells and cause remarkable changes within them.   This influence most likely accounts for the fact that in lesions caused by many viruses intracellular changes assume appearances characteristic enough to be spoken of as inclusion bodies.   In this respect many virus diseases differ from those caused by ordinary bacteria.

INCLUSION BODIES

Viruses usually produce characteristic macroscopic lesions in plants, insects, birds, and animals, or cause marked changes in their condition.   Such alterations and lesions serve as indications of virus activity.   In addition to the characteristic macroscopic lesions already mentioned, many viruses also produce equally characteristic microscopic changes as evidenced by the presence of inclusion bodies in the nuclei and cytoplasm of affected cells. There is no obvious reason why these microscopic changes, in-clusion bodies, should not be used as guides or indicators with the same degree of readiness as that with which the macroscopic lesions are employed.   Diagnoses based upon the pictures pre-sented by aggregates of cells, as in tuberculosis and cancer, are familiar to all and no one is astonished any more at the correct diagnoses made by competent pathologists.   Why not, then, use pathological pictures found within cells as aids in diagnostic and experimental work?   Intracellular pathology can be used in this manner.   As a matter of fact, it has been used for a long time in the diagnosis of rabies and smallpox.

Inclusions have been seen in the cells of plants, insects, fish, birds and mammals affected by virus diseases.   Many of the inclusions described, however, cannot be accepted as specific or characteristic and it is these that detract from the significance of those which are well established and accepted by numerous

critical investigators. No worker familiar with the microscopic pathology of the virus diseases doubts the importance and significance of Guarnieri bodies, Negri bodies, Bollinger bodies, and the nuclear inclusions seen in varicella, herpes, and several other diseases.

Various ideas are held concerning inclusions. Some investigators consider them merely as products of degeneration, but others believe that they are the virus itself, while yet others think of them as virus surrounded by a mantle of altered cellular material. As yet their nature has not been definitely determined. Nevertheless, in spite of the ignorance concerning their nature, inclusion bodies have held and will continue to hold an important position in the study of this group of diseases. Many attempts to produce significant inclusions by artificial means have been unsuccessful. Therefore, under properly controlled conditions the presence of inclusions, accepted as significant, will undoubtedly in the majority of instances be indicative of the presence of a virus in the immediate vicinity.

Inclusion bodies have not been found in all virus diseases. In some they may have been overlooked, while in others they may not occur. A restudy of the diseases of unknown etiology may reveal many interesting changes within the cells. These studies, however, must be made or guided by well trained men with a wide knowledge concerning normal and pathological tissues in order to prevent the literature from being flooded with reports dealing with inclusions not of a characteristic or specific nature.

RESISTANCE TO PHYSICAL AND CHEMICAL AGENTS

It is generally believed that viruses are more resistant to glycerol than are ordinary bacteria. This is not universally true, however. Virus III, the "salivary gland virus" of guinea pigs, and others are not active after remaining in 50 per cent glycerol for six weeks. On the other hand, many bacteria, particularly if in tissues, remain viable in glycerol much longer than 6 weeks. The extensive use of glycerol for the preservation of viruses is

probably largely dependent upon the fact that viruses are usually very susceptible to conditions in autolysing tissues and that glycerol acts as a desiccant and retards autolysis of the tissues containing the viruses.

All viruses are inactivated by high temperatures. The degree of heat necessary to accomplish this varies from 45° to 80°C. depending on the virus. The majority of them resist low temperatures. Repeated freezing and thawing with liquid air (−185°C.), however, does not sharply separate them from ordinary bacteria on the one hand and from enzymes on the other. Under ordinary conditions some retain their activity *in vitro* for periods of 5 years, others become inactive within 48 hours. Some resist putrefaction and drying. Extraction with chloroform, acetone, alcohol, and toluol for periods of 2–8 days does not inactivate some dried viruses (vaccine virus). A few even in a wet state are not inactivated in this manner (virus of tobacco mosaic). Dry spores of certain bacteria (*B. subtilis*) also resist extraction by means of these agents. The virus of contagious epithelioma is active after exposure for twenty-four hours to 1 per cent sodium hydroxide.[3] Three per cent phenol does not inactivate the virus of African horse sickness. Plant cells are supposed to be more tolerant of the injurious action of bile and saponin than are animal cells. In view of the general susceptibility of the viruses to the action of these agents, some workers are inclined to believe that they are more closely related to protozoa than to bacteria. There are a number of striking exceptions to the rule, however. More evidence is not needed to convince one that a wide range in the degree of resistance to physical and chemical agents is exhibited by the viruses, and that a classification based upon resistance to such agents is as impossible of accomplishment as is an adequate classification of ordinary bacteria by means of thermal-death-point determinations.

[3] According to Friedberger, this statement is incorrect.

QUESTION OF THE CORPUSCULAR NATURE OF VIRUSES

The question of the organized or corpuscular nature of the viruses has not been satisfactorily settled. This is due to the fact that most workers realize that the granules which are seen, which are frequently held back by filters, and which are thrown down by prolonged centrifugation may not represent virus alone. This is particularly true since viruses exhibit a remarkable tendency to be adsorbed by many things with which they come in contact.

DO VIRUSES RESPIRE?

Very little information concerning the respiration of viruses is available. Recently, however, Bronfenbrenner reported that he was unable to detect any respiration on the part of phage, herpes virus, and rabies virus in the absence of living host cells. He also found, taking the rate of multiplication into consideration, that growing bacteria plus phage show no more respiratory activity than do growing bacteria alone.

MUTATION (?) OF VIRUSES OR THE RESULTS OF THEIR ADAPTATION TO NEW HOSTS

Mutations of bacteria with concomitant changes in their characteristics are at present of particular interest to bacteriologists. Naturally the question arises then as to whether viruses can mutate. In the field of filterable viruses, however, this is not a new question, inasmuch as it has been under discussion in regard to the relation between vaccine virus and the virus of smallpox since Jenner's time. In spite of all contradictions, it seems that smallpox virus passed through several calves becomes vaccine virus. Furthermore, if a sufficient number of passages is made in calves, it is impossible for this altered virus to regain the characteristics of smallpox virus even after repeated passages in men. Observations of a similar nature have been made in regard to other virus diseases, e.g., contagious epithelioma of chickens and pigeons, and mosaic disease of tobacco and cucumbers. Whether it is correct to speak of these phenomena as

examples of mutation is not known. In any event, when viruses are adapted to alien hosts, their characteristics are frequently altered as well as are those of the diseases produced by them.

DO THE VIRUSES EXIST IN A LIVING STATE?

The question as to whether the viruses are animate or inanimate is also an old one, inasmuch as it was propounded simultaneously with the discovery of the filterable nature of the viruses. Beijerinck's idea of a living contagious fluid called forth many protests. Sanfelice working with fowl-pox in 1914 found that the virus was not inactivated by 1 per cent sodium hydroxide, and, because of this fact, he was led to think of it as an inanimate poison capable of attacking normal cells and producing within them a poison of a similar nature which in turn could attack other normal cells. Thus he described his idea of how a lifeless agent might be passed in series reproducing itself indefinitely. The work of Twort, d'Herelle, Bordet, and others concerning the bacteriophage is familiar to all. The numerous discussions concerning the nature of this active agent have led investigators to question more closely the living nature of other filterable viruses. Many tests have been devised to act as criteria for the presence of life, but so far no one of them has been found satisfactory. Therefore, it is impossible at present to say whether the viruses are animate or inanimate. Furthermore, it is wise to leave the subject at this point as further pursuit of it leads one into the sterile discussion of what life is, a problem still in the realm of metaphysics.

IDENTITY OF THE EPITHELIOTROPIC AND NEUROTROPIC VIRUSES

In table 1 the diseases from swine-pox through rabies have been arranged and bracketed in a way that quickly shows the relation claimed by different workers to exist between members of the group. There is undoubtedly a close relation between the diseases in the upper part of the group-swine-pox through alastrim. It is now generally believed that chicken-pox and smallpox are distinct and different diseases. This has not always

been the case, however, and as late as the middle of the 19th century Hebra taught that they were identical. Even at the present time there is a difference of opinion in regard to the relation of smallpox and varioloid to alastrim on the one hand, and of chicken-pox to alastrim on the other. Gildemeister and Herzberg recently offered experimental evidence to support the idea of a close relation between herpes virus and vaccine virus and suggested that one might be a mutant of the other. Bokay's paper on the relation of chicken-pox to herpes zoster appeared in 1909, and since then a number of other papers have appeared in which the idea that the two diseases are identical has been supported or opposed. For many years there has been much discussion concerning the interrelationship existing between the various kinds of herpes, and this interest has been stimulated by the work of Doerr, Levaditi, Flexner and Amoss, and others on herpes and lethargic encephalitis. There is great confusion of ideas and facts in regard to encephalitis. Several kinds of viruses have been obtained from the brains of individuals who have died after showing signs of encephalitis, and to each of these viruses has been ascribed an etiological rôle. I am not convinced that this rôle, in many instances, is more than an accidental one. Furthermore, since encephalitis follows a number of infectious diseases, I am not convinced that the brain is the proper place to look for the etiological agent, inasmuch as other agents besides viruses and bacteria attack nervous tissue, toxins for instance.

Many of the viruses may be closely related or some may have evolved from a common ancestor. Nevertheless, it will be hard to convince observant workers that chicken-pox, symptomatic herpes, and smallpox now possess much in common. Since a great deal of the evidence in favor of the identity of these viruses has been obtained by means of cross immunity experiments conducted in the skin of human beings and animals, it is possible that a factor generally overlooked or underestimated is responsible for some of the confusion. Jenner and others of his time recognized the fact that skin diseases, exanthems, and extensive mechanical injury might induce a temporary refractory state to vaccine virus. This phenomenon was thought to be dependent

upon some non-specific factor. Ledingham recently reported that India ink injected into the skin of a rabbit rendered it resistant locally to vaccine virus for forty-nine days. Carnot has shown that skin treated several times with ultra-violet light is temporarily refractory to vaccine virus. Experienced workers invariably tell inexperienced ones not to use areas of skin previously handled when conducting cross immunity experiments. Busson has reported that guinea pigs immunized a short time previously to vaccine virus resisted a known lethal dose of rabies virus. He thought that the protection was probably non-specific. Enough has been said to show how easily one may be misled in regard to the identity of the viruses if one is not cognizant of the difficulties usually encountered in this field of work.

MEASLES

Measles is usually placed with the filterable virus diseases. Recent reports, however, support the idea that it does not belong here. Results obtained in its prevention constitute much of the evidence used to substantiate different etiological claims. The conflicting reports concerning the cause and prevention of measles afford an excellent example of the difficulties frequently experienced when one attempts to evaluate work in the virus field. The importance of this disease and the special interest recently aroused concerning it make it advisable to examine in detail some of the reports in the hope of finding an explanation for the diversity of opinions.

Etiology. Blake and Trask, and others have reported that sterile filtrates obtained from measles patients produce lesions in monkeys similar to those seen in man. Tunnicliff, Donges, Ferry and Fisher, and Hibbard and Duval have cultivated a non-hemolytic streptococcus from the blood of measles patients. Tunnicliff recovered the streptococcus in 42 instances from the blood of 52 patients, but from 20 of the cases she was also able to cultivate other bacteria in addition to the streptococcus, e.g., 10 aërobic and 12 anaërobic diphtheroids, 6 filamentous organisms, 4 Gram-negative spirilla, 1 black-pigment-forming bacillus, 4

large spore-forming bacilli, and 3 staphylococci. Furthermore, she was able to obtain a different kind of streptococcus from the blood of patients with German measles. Ferry and Fisher, and Tunnicliff are now able to show that the "measles streptococcus" produces a toxin which may play an important etiological rôle. According to this view there is a striking analogy between measles and scarlet fever. From the blood of measles patients Salimbeni and Kermorgant have cultivated a delicate spirochete associated with a Gram-negative bacillus; Sellards and Bigelow, a small Gram-positive bacillus; Kusama, by means of passage through monkeys, a Gram-positive diphtheroid-like bacillus. Caronia believes that a Gram-negative, anaërobic, filter-passing organism, which he obtained from the blood and several other sources in measles patients, is the cause of the disease. Furthermore, filtrates from Caronia's cultures apparently give negative skin reactions in measles susceptibles and positive ones in individuals who have recovered from the disease.

In spite of the fact that measles is generally considered a virus disease, a great variety of bacteria have been recovered from the blood of measles patients. All of these bacteria cannot be the cause of the disease, however. It seems not unlikely that the blood of measles patients is easily invaded by many kinds of organisms. This fact does not seem very remarkable when one considers the leucopenia and the abnormal condition of all the mucous membranes that regularly accompany the disease.

Prevention. Cenci, in 1907, was probably the first to use convalescent serum in the prevention of measles, and this measure has since been shown to be fairly effective in preventing the disease provided the serum is administered within five or six days after exposure. This type of protection usually lasts only a few weeks. Salisbury, in 1862, reported that subcutaneous injections of wheat rust, a fungus, if made within a few days after exposure, usually prevented the development of measles. Galli, in 1922, reported that injections of normal horse serum protected exposed children. Caronia claims that his vaccine, given within five or six days after exposure, protects as well as convalescent serum. This claim has been confirmed by Nobel

and Schönberger in Pirquet's clinic. They state, however, that the protection is probably non-specific or that it is due to hetero-genetic antibodies. De Gröer and Redlich made a Forssmann antigen from kidney extracts of certain animals. With this antigen they obtained as good, if not better, protection than that secured with convalescent serum. The protection lasted only two to eight weeks. Schilling reported that injection of Caseosan protected children exposed to measles. Tunnicliff immunized goats to the "measles streptococcus." The immune goat serum is said to protect against measles and to exhibit local rash preven-tion propensities. No reports have been seen in regard to the action of normal goat serum. In fact, there is no evidence that such controls were made. By means of virus cultivated in human blood cells and plasma, Degkwitz immunizes the sheep, an ani-mal that has not been shown to be susceptible to measles. This immune sheep serum is reputed to prevent and possibly to cure measles. To act as a preventive it must be administered between the seventh and eleventh days after exposure. This seems odd, since convalescent serum and Caronia's vaccine must be given within five or six days after exposure. Many reports have appeared concerning the action of the immune sheep serum; some favorable, others unfavorable.

The facts presented concerning the work on measles make it obvious that all of the reports cannot be correct. Furthermore, it seems that the successful prevention of the diseases is not always dependent upon specific measures. When one is exposed to measles in the natural way, it is unlikely that one comes in contact with much more than a minimal infecting dose. Under these circumstances there is a possibility that many foreign sub-stances if injected into patients at the proper time after exposure may in a non-specific manner protect them temporarily. This possibility, however, is inadequate excuse for the injection of all kinds of foreign substances into patients, and, if such tactics are continued, it will be difficult to establish the proper measure of prevention when it is found.

MALIGNANT GROWTHS

A discussion of malignant growths in connection with filterable virus diseases may seem out of place. Nevertheless, recent reports concerning the nature and origin of tumors make it necessary to say a word in regard to the subject.

Sanarelli, in 1898, described an infectious myxoma of rabbits. Later Moses was able to transmit the tumor by means of sterile filtrates. Rous and Murphy have shown that some spontaneous tumors in chickens are transmissible by means of tumor filtrates. These chicken tumors have led to a great deal of discussion in regard to their importance in the general study of malignant growths. Recently increased interest has been aroused in the Rous sarcoma and in the general cancer problem by Gye, who reported that two factors are essential for the production of malignant growths, one of which is a living organized virus capable of multiplication in certain complex media. The virus cultivated under these conditions, however, is innocuous and only becomes capable of producing tumors when mixed with a second specific factor found in tumor filtrates inactivated by means of chloroform, heat, and antiseptics. Murphy, Cori, and Harde are of the opinion that Gye did not properly control his experiments. Furthermore, the two former workers have been able to reactivate a chloroform treated filtrate by the addition of substances containing no virus. Since Gye has not conclusively demonstrated that all the virus in his treated filtrates was inactivated and since others have shown that 4 cc. of such filtrates produce tumors when 2 cc. fail, the proof that two factors are essential or that the virus has multiplied in cultures is unconvincing.

Gye's ideas and experimental results concerning the production of chicken sarcomas are diametrically opposed to those of Carrel, who has reported that he was able to produce sarcomas, transmissible by filtrates, by injecting into chickens embryonic tissue mixed with tar, arsenic, or indol. Carrel believes that the etiological agent of these tumors originates within cells of the host under certain conditions and is of a phage-like nature in the sense that it transforms normal cells into malignant ones

which die and liberate more of the active agent. In this manner the virus is supposed to reproduce itself indefinitely. On the other hand, Murphy and Landsteiner were unable to produce sarcomas, transmissible by filtrates, by injecting into chickens mixtures of embryonic tissue and tar. Carrel's conception of the origin of tumors is not a new one, inasmuch as Sanfelice in 1914, Twort, and Doerr more recently voiced exactly the same idea. Nevertheless, no one has offered as much evidence as Carrel that the conception is correct. In view of the fundamental significance of such a conception from a broad biological standpoint, reports concerning attempts to confirm this work will be eagerly awaited by workers in this field. At the present time no final conclusion can be drawn.

FUTURE STUDY OF VIRUS DISEASES

The etiological agents of some of the diseases in table 1 will probably be cultivated or otherwise definitely identified. Consequently attempts to cultivate them should not be abandoned entirely. On the other hand, the indications are that some viruses will not be cultivated in the absence of living cells until cells and their activities can be more accurately imitated. Therefore, cultivation experiments should not engage our entire attention. Kraus and others have suggested that filterable stages of bacteria, protozoa, and spirochetes may play a rôle in certain virus diseases. This is unlikely. The fact that it is impossible to cultivate in simple media the filterable forms of these etiologic agents does not explain why the non-filterable forms are invisible or insusceptible of cultivation.

How, then, is progress to be made in the study of virus diseases? It will be difficult for the best trained workers and doubly difficult for ones poorly trained to progress rapidly in this field. As long as viruses resist cultivation on simple media, just so long will it be necessary to study them in the host, in the tissues of the host, or in emulsions and filtrates of the tissues. At times it may be impracticable to study a disease adequately in its natural host, e.g., man. In such instances efforts are usually

made to establish the disease in a suitable experimental host. In order to recognize and identify the disease in the new host, the worker must be familiar with the clinical and pathological picture in the old one and at the same time capable of employing the necessary immunological tests. Furthermore, it must be remembered that the new host may decidedly alter both the disease picture and the virus, and that this host may be subject also to natural virus diseases of its own. Therefore, under such circumstances, one should be careful in explaining the unknown in the old host by means of facts obtained from studies conducted in the new.

Sufficient attention has not been paid to the effect that viruses have upon each other when acting simultaneously or alternately in the same animal or to the effect that other kinds of diseases have upon the localization and activity of viruses. In regard to herpes zoster, herpes simplex, cancer, and encephalitis following vaccination one frequently hears the statement that a latent virus has become active in the presence of injury or the activity of a new virus. I have already suggested that some viruses might persist in the body for a long time. There is definite proof of this persistence, however, in only a few instances. In how many more it occurs is not known. One should not assume the presence of a latent virus without at least attempting to prove the validity of the assumption.

Prevention of a virus disease does not necessarily depend upon the visibility of the etiological agent or upon a complete knowledge of its pedigree, as is evidenced by vaccination against smallpox and rabies. It appears that the use of active virus either in small amounts or in an attenuated state is the only means by which a lasting protection can be obtained. Money and time would be well spent, then, in attempting to remove the unnecessary and objectionable ingredients of virus emulsions used for vaccination purposes. Moreover, it might be wise to make further attempts to determine whether, in some instances, instead of vaccination with active viruses, the repeated subcutaneous injections of purified and inactivated viruses lead to a degree of immunity sufficient to warrant their use.

SUMMARY

In the majority of virus diseases a close relationship between the etiological agents and the cells of the hosts exists. This intimate type of parasitism is emphasized by the fact that some of the diseases exhibit a striking species specificity, that the viruses have resisted cultivation in the absence of living cells, that characteristic or specific pathological changes are frequently observed in cells affected by viruses, and, finally, that a host once recovered from a virus disease usually exhibits a lasting immunity.

REFERENCES

General reviews and discussions

(1) BAYON, H. P.: Jour. Trop. Med. and Hyg., 1926, **29**, 17.
(2) DOERR, R.: Centr. Bak., Beilage, Abt. 1, Ref., 1911, **50**, *12.
(3) KRAUS, R.: Med. Klinik, 1926, **22**, 540, 579.
(4) LOEFFLER, F.: Centr. Bak., Beilage, Abt. 1, Ref., 1911, **50**, *1.
(5) LUCKSCH, F.: Prag. Arch. Tiermed., Teil A, 1925, **5**, 83.
(6) MacCALLUM, W. G.: Medicine, 1926, **5**, 59.
(7) M'FADYEAN, J.: Jour. Comp. Path. and Therap., 1908, **21**, 58, 168, 232.
(8) PHILBERT, A.: Anns. Méd., 1924, **16**, 283.
(9) SIMON, C. E.: Physiological Reviews, 1923, **3**, 483.
(10) ROUX, E.: Bull. Inst. Pasteur, 1903, **1**, 7, 49.
(11) WOLBACH, S. B.: Jour. Med. Research, 1912, **27**, 1.
(12) TWORT, F. W.: Jour. State Med., 1923, **31**, 351.

Filtration and size

(13) ANDRIEWSKY, P.: Centr. Bak., Abt. 1, Orig., 1915, **75**, 90.
(14) BARNARD, J. E.: Lancet, 1925, **2**, 117.
(15) BECHHOLD, H., AND VILLA, L.: Biochem. Zt., 1925, **165**, 250; Centr. Bak., Abt. 1, Orig., 1926, **97**, *162; Zt. Hyg., 1926, **105**, 601.
(16) D'HERELLE, F.: Centr. Bak., Abt. 1, Orig., 1925, **96**, 385.
(17) DUGGAR, B. M., AND KARRER, J. L.: Anns. Missouri Bot. Gard., 1921, **8**, 343.
(18) DUGGAR, B. M., AND ARMSTRONG, J. K.: Anns. Missouri Bot. Gard., 1923, **10**, 191.
(19) DuNoüy, P. L.: Surface Equilibria of Colloids, N. Y., 1926 (Amer. Chem. Soc. monograph series).
(20) GROLLMAN, A.: Jour. Gen. Physiol., 1926, **9**, 813.
(21) HOLMAN, W. L.: Amer. Jour. Path., 1926, **2**, 483.
(22) KRAMER, S. P.: Jour. Gen. Physiol., 1926, **9**, 811.
(23) KRAMER, S. P.: Science, 1927, **65**, 45.

(24) KRAUS, R.: Centr. Bak., Abt. 1, Orig., 1926, 97, *160.
(25) MUDD, S., AND MUDD, E. B. H.: Jour. Bact., 1924, 9, 151.

Cultivation of viruses in tissue cultures: A review

(26) MEYER, E.: Arch. exp. Zellforschung, 1926, 3, 201.

Inclusions (reviews and discussions)

(27) FINDLAY, G. M., AND LUDFORD, R. J.: Brit. Jour. Exp. Path., 1926, 7, 223.
(28) LIPSCHÜTZ, B.: Wein. klin. Woch., 1919, 32, 851, 1127; Centr. Bak., Abt. 1,
        Orig., 1922, 87, 303; 1925, 96, 222; Seuchenbekämpfung, 1926, 3, 79.
(29) LOEWENTHAL, W.: Centr. Bak., Abt. 1, Orig., 1926, 97, *139.
(30) LUGER, A., AND LAUDA, E.: Med. Klin., 1926, 22, 415, 456, 493; Centr. Bak.,
        Abt. 1, Orig., 1926, 97, *135.
(31) LUGER, A., AND SILBERSTERN, E.: Zt. gesam. exp. Med., 1925, 47, 545.

Filterable stages of bacteria, protozoa, and spirochetes

(32) ALMQUIST, E.: Centr. Bak., Abt. 1, Orig., 1911, 60, 167.
(33) BURNET, E.: Compt. rend. Soc. biol., 1926, 95, 1142.
(34) CALMETTE, A., AND VALTIS, J.: Anns. Méd., 1926, 19, 553.
(35) DURAND, H., AND VAUDREMER, A.: Compt. rend. Soc. biol., 1924, 90, 916
(36) FONTES, A.: Mem. Inst. Oswaldo Cruz, 1910, 2, 141.
(37) HAUDUROY, P.: Paris Méd., 1916, 59/60, 544.
(38) KRAUS: Centr. Bak., Abt. 1, Orig., 1926, 97, *160.
(39) VALTIS, J.: Anns. Inst. Pasteur, 1924, 38, 453.

*Effects of mixtures of viruses. Other kinds of diseases in relation to the
localization and activity of viruses*

(40) BELIN, M.: Compt. rend. Soc. biol., 1925, 93, 1488; 1926, 94, 387, 816.
(41) DENNEY, O. E., AND HOPKINS, R.: Pub. Health Rep., 1922, 37, 3141.
(42) DICKSON, B. T.: Science, 1925, 62, 398.
(43) FERNOW, K. H.: N. Y. Agric. Exp. Sta. Mem., 1925, No. 96, 1–34.
(44) HASSELTINE, H. E.: Pub. Health Rep., 1923, 38, 1.
(45) JOHNSON, J.: Wisconsin Agric. Exp. Sta. Bull., 1925, No. 63, 1-12.
(46) LEVADITI, C., AND NICOLAU, S.: Compt. rend. Soc. biol., 1925, 93, 3.
(47) REMLINGER, P., AND BAILLY, J.: Compt. rend. Soc. biol., 1925, 93, 1486.
(48) TILLETT, W. S., AND RIVERS, T. M.: Bull. Johns Hopkins Hosp., 1924, 35,
        137.
(49) VANTERPOOL, T. C.: Phytopathology, 1926, 16, 311.

Malignant growths

(50) BORREL, A.: Anns. Inst. Pasteur, 1903, 17, 81; Compt. rend. Soc. biol.,
        1904, 57, 642.
(51) CARREL, A.: Jour. Amer. Med. Assoc., 1925, 84, 1795; Compt. rend. Soc.
        biol., 1925, 93, 1083, 1278; 92, 1493.
(52) CORI, C. F.: Proc. Soc. Exp. Biol. and Med., 1926, 24, 65.

(53) DOERR, R.: Klin. Woch., 1922, **1**, 1489, 1537.

(54) FISCHER, A.: Compt. rend. Soc. biol., 1926, **94**, 1217.

(55) GYE, W. E.: Lancet, 1925, **2**, 109; 1926, **2**, 989.

(56) HARDE, E.: Jour. Trop. Med. and Hyg., 1926, **29**, 159.

(57) MOSES, A.: Mem. Inst. Oswaldo Cruz, 1911, **3**, 46.

(58) MURPHY, J. B., AND LANDSTEINER, K.: Jour. Exp. Med., 1925, **41**, 807.

(59) MURPHY, J. B.: Jour. Amer. Med. Assoc., 1926, **86**, 1270.

(60) ROUS, P.: Jour. Exp. Med., 1911, **13**, 397.

(61) ROUS, P., AND MURPHY, J. B.: Jour. Exp. Med., 1914, **20**, 419; Jour. Amer. Med. Assoc., 1912, **58**, 1751.

(62) SANARELLI, G.: Centr. Bak. Abt. 1, 1898, **23**, 865.

(63) SIMON, C. E., AND BECK, M. D.: Amer. Jour. Hyg., 1926, **6**, 659.

Bacteriophage

(64) BORDET, J.: British Med. Jour., 1922, **2**, 296.

(65) BRONFENBRENNER, J. J.: Science, 1926, **63**, 51; Proc. Soc. Exp. Biol. and Med., 1926, **23**, 635.

(66) BRONFENBRENNER, J. J., AND REICHERT, P.: Proc. Soc. Exp. Biol. and Med., 1926, **24**, 176

(67) D'HERELLE, F.: British Med. Jour., 1922, **2**, 289.

(68) D'HERELLE, F.: The Bacteriophage and Its Behavior, Baltimore (English), 1926.

(69) GRATIA, A.: British Med. Jour., 1922, **2**, 296.

(70) HADLEY, P.: Jour. Infect. Dis., 1925, **37**, 35.

(71) RIVERS, T. M.: Jour. Exp. Med., 1927, **45**, 11.

(72) TWORT, F. W.: Lancent, 1915, **2**, 1241; 1925, **2**, 642; Brit. Med. Jour., 1922, **2**, 293.

Mosaic diseases (infectious chloroses)

(73) ALLARD, H. A.: Bull. U. S. Dept. Agric., 1914, No. 40; Jour. Agric. Research, 1916, **6**, 649; 1917, **10**, 615.

(74) BAUR, E.: B. d. deut. Bot. Gesellsch., 1904, **22**, 453; 1906, **24**, 416; 1907, **25**, 410.

(75) BEIJERINCK, M. W.: Centr. Bak. Abt. II, 1899, **5**, 27, 310.

(76) BRANDES, E. W.: Jour. Agric. Research, 1923, **23**, 279; Bull. U. S. Dept. Agric., 1919, No. 829.

(77) BRANDES, E. W., AND KLAPHAAK, P. J.: Jour. Agric. Research, 1923, **24**, 247.

(78) CARSNER, E., AND STAHL, C. F.: Jour. Agric. Research, 1924, **28**, 297.

(79) DOOLITTLE, S. P.: Bull. U. S. Dept. Agric., 1920, No. 879; Circular U. S. Dept. Agric., 1924, No. 321.

(80) DUGGAR, B. M., AND KARRER, J. L.: Anns. Missouri Bot. Gard., 1921, **8**, 343.

(81) DUGGAR, B. M., AND ARMSTRONG, J. K.: Anns. Missouri Bot. Gard., 1923, **10**, 191, 1925, **12**, 359.

(82) GARDNER, M. W.: Jour. Agric. Research, 1925, **30**, 871.

(83) IWANOWSKI, D.: Zt. Pflanzenkrank., 1903, **13**, 1; Centr. Bak., Abt. II, 1899, **5**, 250.

(84) JAGGER, I. C.: Jour. Agric. Research, 1920–21, **20**, 737.
(85) McKINNEY, H. H Jour. Agric. Research, 1923, **23**, 771.
(86) McKINNEY, H. H., AND ECKERSON, S. H.: Jour. Agric. Research, 1923, **26**, 605.
(87) McKINNEY, H. H.: Bull. U. S. Dept. Agric., 1925, No. 1361.
(88) MULVANIA, M.: Science, 1925, **62**, 37.
(89) OLITSKY, P. K.: Jour. Exp. Med., 1925, **41**, 129.
(90) PURDY, H. A.: Botanical Gazette, 1926, **81**, 210.
(91) PALM, B. T.: Centr. Bak., Abt. II, 1923–24, **60**, 215.
(92) RAWLINS, T. E., AND JOHNSON, J.: Amer. Jour. Bot., 1925, **12**, 19.

Virus diseases of insects

(93) GLASER, R. W.: Jour. Agric. Research, 1915, **4**, 101.
(94) PAILLOT, A.: Anns. Inst. Pasteur, 1926, **40**, 314.
(95) VON PROWAZEK, S.: Centr. Bak., Abt. 1, Orig., 1913, **67**, 268.
(96) WHITE, G. F.: Bull. U. S. Dept. Agric., 1917, No. 431.

Epizoötic of guinea pigs

(97) PETRIE, G. F., AND O'BRIEN, R. A.: Jour. Hyg., 1910, **10**, 287.
(98) SANGIORGI, G.: Centr. Bak., Abt. 1, Orig., 1914, **72**, 70.

Hog cholera

(99) HUTYRA, F., AND MAREK, J.: Pathologie und Therapie der Haustiere, 4th ed., Jena, 1913, vol. i, p. 259.
(100) UHLENHUTH, P.:Centr. Bak. (Festsch. Loeffler) 1912, **64**, 151.

Cattle plague

(101) HUTYRA, F., AND MAREK, J.: Pathologie und Therapie der Haustiere, 4th ed., Jena, 1913, vol. i, 237.
(102) MINETT, F. C.: Jour. Comp. Path. and Therap., 1923, **36**, 205.
(103) NICOLLE, M., AND ADIL-BEY: Anns. Inst. Pasteur., 1902, **16**, 56.

Pernicious anemia of horses

(104) DeKOCK, G. v. d. W.: Union of South Africa, Dept. Agric., 9th and 10th Report. Director Vet. Research., 1923, 253. (Abstracted in Trop. Vet. Bull., 1924, **12**, 136.
(105) TODD, J. L., AND WOLBACH, S. B.: Jour. Med. Research, 1911, **24**, 213.
(106) VALLÉE AND CARRÉ: Compt. rend. Acad. Sc., 1904, **139**, 331, 1239.

Virus III infection of rabbits

(107) ANDREWES, C. H., AND MILLER, C. P., Jr.: Jour. Exp. Med., 1924, **40**, 789.
(108) MILLER, C. P., Jr., ANDREWES, C. H., AND SWIFT, H. F.: Jour. Exp. Med., 1924, **40**, 773.
(109) RIVERS, T. M., AND TILLETT, W. S.: Jour. Exp. Med., 1923, **38**, 673; 1924, **39**, 777; **40**, 281.
(110) RIVERS, T. M., AND PEARCE, L.: Jour. Exp. Med., 1925, **42**, 523.

Foot-and-mouth disease

(111) FROSCH: Berlin. tierärzt. Woch., 1924, **40**, 341.
(112) FROSCH, AND DAHMEN, H.: Berlin. tierärzt. Woch., 1924, **40**, 185, 273, **662**.
(113) GUTH, F.: Deut. med. Woch., 1924, **50**, 707.
(114) LEBAILLY, C.: Compt. rend. Acad. Sc., 1926, **183**, 578.
(115) LOEFFLER AND FROSCH: Centr. Bak., Abt. 1, 1897, **22**, 257, 1898, **23**, 371.
(116) STOCKMAN, S., AND MINETT, F. C.: Jour. Comp. Path. and Therap., 1926, **39**, 231.
(117) VALLÉE, H., AND CARRÉ, H.: Compt. rend. Acad. Sc., 1922, **174**, 207, 1498.

Vesicular stomatitis of horses and cattle

(118) OLITSKY, P. K., TRAUM, J., AND SCHOENING, H. W.: Jour. Amer. Vet. Med. Assoc., 1926, **70**, 147.

Paravaccinia

(119) LIPSCHÜTZ, B.: Centr. Bak., Abt. 1, Orig., 1918, **81**, 105; Wien. klin. Woch., 1918, **31**, 93.
(120) VON PIRQUET, C.: Zt. Kinderheilk., 1916, **13**, 309.

Smallpox, alastrim, and pox diseases of animals

(121) BACHMANN, A., AND BIGLIERI, R.: Compt. rend. Soc. biol., 1923, **88**, 350.
(122) BUSSON, B.: Wien. klin. Woch., 1926, **39**, 1183.
(123) CALAMETTE, A., AND GUÉRIN, C.: Anns. Inst. Pasteur, 1901, **15**, 161.
(124) CARNOT, P., CAMUS, L., AND BENARD, H.: Comp. rend. Soc. biol., 1926, **95**, 457.
(125) COPEMAN, S. M.: Jour. Path. and Bact., 1894, **2**, 407; Proc. Roy. Soc., London, 1903, **71**, 121.
(126) COPLANS, M.: Jour. Trop. Med. and Hyg., 1926, **29**, 122, 133.
(127) COWDRY, E.V.: Jour. Exp. Med., 1922, **36**, 667.
(128) CRACIUN, E. C., AND OPPENHEIMER, E. H.: Bull. Johns Hopkins Hosp., 1925, **37**, 428.
(129) CROOKSHANK, E. M.: History and Pathology of Vaccination, vols. i and ii, Philadelphia, 1889.
(130) FORNET, W.: Deut. med. Woch., 1913, **39**, 1813; Centr. Bak., Abt. 1, Orig., 1922, **87**, 36.
(131) EWING, J.: Jour. Med. Research, 1904–05, **13**, 233.
(132) GILDEMEISTER, E., AND HERZBERG, K.: Deut. med. Woch., 1925, **51**, 1647.
(133) GINS, H. A.: Zt. Hyg., 1916, **82**, 89; 1919, **89**, 231; 1920, **90**, 322.
(134) GORDON, M. H.: Medical Research Council, Special Report Series, No. 98, London, 1925.
(135) GUARNIERI, G.: Centr. Bak., 1894, **16**, 299.
(136) HACH, I. W.: Centr. Bak., Abt. 1, Orig., 1925, **94**, 270.
(137) HARDE, E. S.: Compt. rend. Soc. biol., 1915, **78**, 545; Anns. Inst. Pasteur, 1916, **30**, 299.
(138) JENNER, E.: Medical and Physical Jour., 1804, **12**, 97.

(139) JENNER, E.: Appendix No. 1, On Vaccine Inoculation, Robert Willan, London, 1806.
(140) JORGE, R.: Lancet, 1924, 2, 1317, 1366.
(141) LEDINGHAM, J. C. G.: Brit. Jour. Exp. Path., 1924, 5, 332; Jour. Path. and Bact., 1926, 29, 309; Lancet, 1925, 1, 199.
(142) LEVADITI, C.: Jour. State Medicine, London, 1924, 32, 151.
(143) LEVADITI, C., AND NICOLAU, S.: Anns. Inst. Pasteur, 1923, 37, 443; Compt. rend. Acad. Sc., 1921, 173, 870.
(144) LEVADITI, C., HARVIER, P., AND NICOLAU, S.: Compt. rend. Soc. biol., 1921, 85, 345.
(145) MACCALLUM, W. G., AND MOODY, L. M.: Amer. Jour. Hyg., 1921, 1, 388.
(146) MINERVIN, S., AND SCHMERLING, A.: Centr. Bak., Abt. 1, Orig., 1926, 99, 558.
(147) NAKAGAWA, S.: Zt. Immunitäts, Orig., 1924, 39, 173, 563.
(148) NOGUCHI, H.: Jour. Exp. Med., 1915, 21, 539; 1918, 27, 425.
(149) OHTAWARA, T.: Japan Med. World, 1922, 2, 254; 1923, 3, 1.
(150) PARKER, F., JR.: Jour. Med. Research, 1924, 44, 645.
(151) PARKER, F., JR., AND NYE, R. N.: Amer. Jour. Path., 1, 325
(152) SCHÜTZ, V.: Zt. Hyg., 1926, 105, 1.
(153) STEINHARDT, E., ISRAELI, C., AND LAMBERT, R. A.: Jour. Infect. Dis., 1913, 13, 294.
(154) STEINHARDT, E., AND LAMBERT, R. A.: Jour. Infect. Dis., 1914, 14, 87.
(155) TSURUMI, M., TOYODA, T., AND INOUYE, T.: Japan Med. World, 1922, 2, 221.
(156) TURKHUD, D. A., AND PANDIT, C. G.: Indian Jour. Med. Research, 1926, 14, 27.
(157) TYZZER, E. E.: Jour. Med. Research, 1904, 11, 180.
(158) VAN HOOF, L.: Anns. Soc. Belge Méd. Trop., 1923–24, 3, 377; 1925, 5, 1.
(159) VON PROWAZEK, S., AND MIYAJI, S.: Centr. Bak., Abt. 1, Orig., 1915, 75, 144.
(160) VON PROWAZEK, S.: v. Prowazek, Handbuch der pathogenen Protozoen, 1912, Leipzig, 1 and 2, pp. 122 and 139.
(161) VON WASIELEWSKI, T., AND WINKLER, W. F.: Ergeb. Hyg., Bak., Immunitäts. u. exp. Therap., 1925, 7, 1.
(162) WILLAN, R.: On vaccine inoculation, London, 1806, page 34.
(163) WINKLER, W. F.: Ergeb. allgem. Path. u. path. Anat., Abt. 1, 1925, 21, 45.

Chicken-pox and herpes zoster

(164) COLE, R., AND KUTTNER, A. G.: Jour. Exp. Med., 1925, 42, 799.
(165) DOERR, R.: Zentr. Haut-u. Geschlechtskrankh., 1924, 13, 417; 15, 1, 129, 289; 1925, 16, 481.
(166) HEBRA, F.: On diseases of the skin including the exanthemata, New Sydenham Society, London, 1864, 1, 230.
(167) KLING, C. A.: Berl. klin. Woch., 1913, 50, 2083; 1915, 52, 13.
(168) KUNDRATITZ, K.: Zt. Kinderheilk., 1925, 39, 379.
(169) KUNDRATITZ, K.: Monatschr. Kinderheilk., 1924–25, 29, 516.
(170) DE LANGE, C.: Klin. Woch., 1923, 2, 879.
(171) LAUDA, E., AND SILBERSTERN, E.: Klin. Woch., 1925, 4, 1871.

(172) LAUDA, E., AND STÖHR, D.: Monatschr. Kinderheilk., 1926, **34**, 97.
(173) LESNÉ AND DE GENNES: Bull. Soc. méd. Hôp., Paris, 1925, **49**, 221.
(174) LIPSCHÜTZ, B., AND KUNDRATITZ, K.: Wien. klin. Woch., 1925, **38**, 499.
(175) LIPSCHÜTZ, B.: Centr. Bak., Abt. 1, Orig., 1924, **93**, 361.
(176) LOW, R. C.: British Med. Jour., 1919, **1**, 91.
(177) NETTER, A.: Bull. Acad. Méd., 1924, **91**, 494.
(178) NETTER, A.: Bull. Soc. méd. Hôp., Paris, 1925, **49**, 249.
(179) NETTER, A., AND URBAIN, A.: Compt. rend. Soc. biol., **90**, 461.
(180) NETTER, A., AND URBAIN, A.: Compt. rend. Soc. biol., 1926, **94**, 98.
(181) PETZETAKIS: Bull. Soc. méd. Hôp., Paris, **49**, 1000.
(182) RIVERS, T. M.: Jour. Exp. Med., 1926, **43**, 275.
(183) SIMON, C. E., AND SCOTT, J. M.: Amer. Jour. Hyg., 1924, **4**, 675.
(184) STEINER: Wein. med. Woch., 1875, **25**, 306.
(185) TEAGUE, O., AND GOODPASTURE, E. W.: Jour. Med. Research, 1923–24, **44**, 185.
(186) VON BÓKAY, J.: Wein. klin. Woch., 1909, **22**, 1323; Jahrb. Kinderheilk, 1924, **105**, 8.
(187) WEECH, A. A.: Jour. Amer. Med. Assoc., 1924, **82**, 1245.

Symptomatic herpes (herpes simplex) and lethargic encephalitis

(188) DOERR, R.: Centr. Bak., Abt. 1, Orig., 1926, **97**, *76.
(189) DOERR, R.: Klin. Monatsbl. Augenheilk, 1920, **65**, 104.
(190) DOERR, R., AND SCHNABEL, A.: Zt. Hyg., 1921, **94**, 29.
(191) DOERR, R., AND VÖCHTING, K.: Rev. gén. ophth., 1920, **34**, 409.
(192) DOERR, R., AND ZDANSKY, E.: Zt. Hyg., 1924, **102**, 1.
(193) V. ECONOMO, C.: Wien. klin. Woch., 1917, **30**, 581.
(194) FLEXNER, S.: Jour. Amer. Med. Assoc., 1923, **81**, 1688, 1785.
(195) FLEXNER, S., AND AMOSS, H. L.: Jour. Exp. Med., 1925, **41**, 215, 233, 357.
(196) GOOD PASTURE, E. W.: Amer. Jour. Path., 1925, **1**, 1, 11, 29, 47.
(197) GOODPASTURE, E. W., AND TEAGUE, O.: Jour. Med. Research, 1923–24, **44**, 121, 139.
(198) GRÜTER, W.: Ber. 42. Versamml. ophth. Ges., 1920, 162; Klin. Monatsbl. Augenheilk., 1920, **65**, 398.
(199) HOFF, H., AND SILBERSTEIN, F.: Zt. gesam. exp. Med., 1924–25, **44**, 268.
(200) JAHNEL: Centr. Bak., Abt. 1, Orig., 1926, **97**, *151.
(201) LAUDA, E., AND LUGER, A.: Ergeb. inner. Med. u. Kinderheil., 1926, **30**, 377.
(202) LEVADITI, C., AND HARVIER, P.: Anns. Inst. Pasteur, 1920, **34**, 911.
(203) LIPSCHÜTZ, B.: Arch. Dermatol. u. Syph., Orig., 1921, **136**, 428.
(204) LÖWENSTEIN, A.: Münch. med. Woch., 1919, **66**, 769; Klin. Monatsbl. Augenheilk, 1920, **64**, 15.
(205) LUGER, A., AND LAUDA, E.: Wien. klin. Woch., 1925, **38**, 33.
(206) PERDRAU, J. R.: Brit. Jour. Exp. Path., 1925, **6**, 41, 123.
(207) ROSENOW, E. C.: Arch. Neurol. and Psychiat., 1926, **15**, 712.
(208) STERN, F.: Centr. Bak., Abt. 1, Orig., 1926, **97**, *94.
(209) TAKAKI, J., AND OTHERS: Zt. Immunitäts, 1926, **47**, 431.
(210) WRIGHT, J. H., AND CRAIGHEAD, E. M.: Jour. Exp. Med., 1922, **36**, 135.
(211) ZINSSER, H., AND TANG, F.: Jour. Exp. Med., 1926, **44**, 21.

Postvaccinal encephalitis

(212) Bastiaanse, F. S. van B., et al.: Nederland Tijdsch. v. Geneesk., 1926, **20**, 1267.

(213) Blanc, G., and Caminopetros, J.: Schweiz. med. Woch., 1926, **7**, 131.

(214) Comby, J.: Bull. Soc. méd. Hôp., Paris, 1926, **50**, 1434.

(215) Frommel, E., and Baumgartner, J.: Schweiz. med. Woch., 1926, **7**, 857.

(216) Kraus, R., and Takaki, J.: Med. Klinik, 1925, **21**, 1872.

(217) Levaditi, C., and Nicolau, S.: Compt. rend. Soc. biol., 1926, **94**, 114.

(218) Lucksch, F : Med. Klinik, 1925, **21**, 1377; Centr. Bak., Abt. 1, Orig., 1925, **96**, 309.

(219) Turnbull, H. M., and McIntosh, J.: Brit. Jour. Exp. Path., 1926, **7**, 181.

(220) Walthard, B.: Schweiz. med. Woch., 1926, **7**, 854.

(221) Winkler, W. F.: Deut. med. Woch., 1926, **52**, 477.

Encephalitis in Japan

(222) Kaneko, R.: Japan Med. World, 1925, **5**, 237.

(223) Kobayashi, R.: Japan Med. World, 1925, **5**, 145.

(224) Takagi, I.: Japan Med. World, 1925, **5**, 147; Zt. Immunitäts., 1926, **47**, 441, 456.

Koritschoner virus

(225) Koritschoner, R.: Münch. med. Woch., 1923, **70**, 896; Viroh. Arch., 1925, **255**, 172.

Australian X disease

(226) Kneebone, J. LeM., and Cleland, J. B.: Austral. Jour. Exp. Biol., 1926, **3**, 119.

Poliomyelitis

(227) Amoss, H. L.: Bull. N. Y. Acad. Med., 1926, 2nd series, **2**, 456; Jour. Exp. Med., 1917, **25**, 545.

(228) Bull, C. G.: Jour. Exp. Med., 1917, **25**, 557.

(229) Flexner, S., and Lewis, P. A.: Jour. Amer. Med. Assoc., 1909, **53**, 1639, 1913, 2095; 1910, **54**, 1780.

(230) Flexner, S., and Noguchi, H.: Jour. Amer. Med. Assoc., 1913, **60**, 362; Jour. Exp. Med., 1913, **18**, 461.

(231) Flexner, S., Noguchi, H., and Amoss, H. L.: Jour. Exp. Med., 1915, **21**, 91.

(232) Landsteiner, K., and Popper, E.: Zt. Immunitäts, 1909, **2**, 377.

(233) Landsteiner, K., and Levaditi, C.: Compt. rend. Soc. biol., 1909, **67**, 592, 787.

(234) Levaditi, C., and Landsteiner, K.: Compt. rend. Soc. biol., 1910, **68**, 311.

(235) Levaditi, C.: Compt. rend. Soc. biol., 1913, **74**, 1179; **75**, 202.

(236) Rosenow, E. C., and Wheeler, G. W.: Jour. Infect. Dis., 1918, **22**, 281.

(237) Rosenow, E. C., Towne, E. B., and v. Hess, C. L.: Jour. Infect. Dis., **22**, 313.

(238) Rosenow, E. C., and Gray, H.: Jour. Infect. Dis., 1918, **22**, 345.

(239) Sherwood, N. P., and Downs, C. M.: Jour. Infect. Dis., 1919, **24**, 133.

(240) Smillie, W. G.: Jour. Exp. Med., 1918, **27**, 319.

Rabies

(241) v. EISLER, M.: Centr. Bak., Abt. 1, Orig., 1908, **45**, 71.
(242) FERMI, C.: Senchenbekämpfung, 1926, **3**, 114.
(243) GALTIER: Compt. rend. Acad. Sc., 1879, **89**, 444.
(244) GOODPASTURE, E. W.: Amer. Jour. Path., 1925, **1**, 547.
(245) LEVADITI, C.: Compt. rend. Soc. biol., 1913, **75**, 505.
(246) LEVADITI, C., NICOLAU, S., AND SCHOEN, R.: Anns. Inst. Pasteur, 1926, **40**, 973.
(247) LUBINSKI, H.: Klin. Woch., 1926, **5**, 1419.
(248) NEGRI, A.: Zt. Hyg., 1903, **43**, 507; **44**, 519; 1909, **63**, 421.
(249) NOGUCHI, H.: Jour. Exp. Med., 1913, **18**, 314.
(250) PASTEUR, L., CHAMBERLAND, AND ROUX: Compt. rend. Acad. Sc., 1881, **92**, 159.
(251) REMLINGER, P.: Anns. Inst. Pasteur, 1903, **17**, 834; 1904, **18**, 150.
(252) STEINHARDT, E., POOR, D. W., AND LAMBERT, R. A.: Jour. Infect. Dis., 1912, **11**, 459.

Borna's disease

(253) JOEST, E.: Ergeb. allgem. Path. u. path. Anat., 1915, **18**, Abt. 1, 359.
(254) JOEST, E.: Klin. Woch., 1926, **5**, 209.
(255) ZWICK, W.: Senchenbekämpfung, 1926, **3**, 57.
(256) ZWICK, W., SEIFRIED, O., AND WITTE, J.: Zt. Infektionskrank. Haustiere, 1926, **30**, 42.

Fowl plague and plague of blackbirds

(257) CENTANNI, E.: Centr. Bak., Abt. 1, Orig., 1902, **31**, 145, 182.
(258) KLEINE, F. K.: Zt. Hyg., 1905, **51**, 177.
(259) LANDSTEINER, K., AND BERLINER, M.: Centr. Bak., Abt. 1, Orig., 1913, **67**, 165.
(260) MAGGIORA, A., AND VALENTI, G. L.: Zt. Hyg., 1903, **42**, 185; Centr. Bak., Abt. 1, Orig., 1903, **34**, 326.
(261) MARCHOUX, E.: Compt. rend. Acad. Sc., 1908, **147**, 357.
(262) MROWKA: Centr. Bak., Abt. 1, Orig., 1913, **67**, 249.
(263) OTTOLENGHI, D.: Centr. Bak., Abt. 1, Orig., 1913, **67**, 510.
(264) SCHIFFMAN, J.: Wien. klin. Woch., 1906, **19**, 1347.

Guinea pig paralysis

(265) RÖMER, P. H.: Deut. med. Woch., 1911, **37**, 1209; Centr. Bak., Beilage, Abt. 1, Ref., 1911, **50**, *30.

Distemper of dogs

(266) DUNKIN, G. W., AND LAIDLAW, P. P.: Jour. Comp. Path. and Therap., 1926, **39**, 201, 213.
(267) KANTOROWICZ, R.: Berl. tierärztl. Woch., 1926, **42**, 185.
(268) LAIDLAW, P. P., AND DUNKIN, G. W.: Jour. Comp. Path. and Therap., 1926, **39**, 222.

Trachoma and inclusion blenorrhea

(269) HALBERSTAEDTER, L.: v. Prowazek, Handbuch der pathogenen Protozoen, 1912, Leipzig, 1 and 2, p. 172.
(270 LINDNER, K.: Deut. med. Woch., 1910, 36, 1326; Centr. Bak., Beilage, Abt. 1, Ref., 1911, 50, *23.
(271) NOGUCHI, H., AND COHEN, M.: Jour. Exp. Med., 1913, 18, 572.

Infectious papular stomatitis of cattle

(272) OSTERTAG AND BUGGE: Zt. Infectionskrank. Haust., 1905–1906, 1, 3.

Molluscum contagiosum

(273) KINGERY, L. B.: Arch. Dermatol. and Syph., 1920, 2, 144.
(274) KNOWLES, F. C.: Jour. Amer. Med. Assoc., 1909, 53, 671.
(275) DA ROCHA-LIMA, H.: v. Prowazek, Handbuch der pathogenen Protozoen, 1920, Leipzig, 2, p. 969.
(276) WHITE, C. J., AND ROBEY, W. H.: Jour. Med. Research, 1902, n.s. 2, 255.

Warts

(277) KINGERY, L. B.: Jour. Amer. Med. Assoc., 1921, 76, 440.
(278) WILE, U. J., AND KINGERY, L. B.: Jour. Amer. Med. Assoc., 1919, 73, 970.

Contagious epithelioma

(279) ANDERVONT, H. B.: Amer. Jour. Hyg., 1926, 6, 719.
(280) BOLLINGER, O.: Virch. Arch., 1873, 58, 349.
(281) FRIEDBERGER, E.: Zt. Immunitäts., 1918, 27, 459.
(282) LIPSCHÜTZ, B.: v. Prowazek, Handbuch der pathogenen Protozoen, 1912, Leipzig, 1 and 2, p. 231.
(283) LISBOA, H. M.: Mem. Inst. Oswaldo Cruz, 1922, 15, 77.
(284) LOEWENTHAL, W., KADOWAKI, Y., AND KONDO, S.: Centr. Bak., Abt. 1, Orig., 1925, 94, 185.
(285) LUSENA, M.: Zt. Hyg., 1926, 106, 65.
(286) MARX, E., AND STICKER, A.: Deut. med. Woch., 1902, 28, 893.
(287) SANFELICE, F.: Zt. Hyg., 1914, 76, 257.
(288) TOYODO, T.: Zt. Hyg., 1924, 102, 592.
(289) VAN HEELSBERGEN, T.: Centr. Bak., Abt. 1, Orig., 1920, 84, 288; 1923, 89, 173.

Leukemia of chickens

(290) ELLERMAN, V., AND BANG, O.: Centr. Bak., Abt. 1, Orig., 1908, 46, 595.

Diseases of fish

(291) WEISSENBERG, R.: v. Prowazek, Handbuch der pathogenen Protozoen, 1921, Leipzig, 3, p. 1344.
(292) KEYSSELITZ, G.: Arch. Protistenkunde, 1908, 11, 326.
(293) LOEWENTHAL, W.: Zt. Krebsforch., 1907, 5, 197.

Mumps

(294) KERMORGANT, Y.: Anns. Méd., 1926, **19**, 301.
(295) KERMORGANT, Y.: Anns. Inst. Pasteur, 1925, **39**, 565.
(296) NICOLLE, C., AND CONSEIL, E.: Compt. rend. Acad. Sc., 1913, **157**, 340.
(297) WOLLSTEIN, M.: Jour. Exp. Med., 1916, **23**, 353.

Agalactia

(298) BRIDRÉ, J., AND DONATIEN, A.: Compt. rend. Acad. Sc., 1923, **177**, 841.
(299) BRIDRÉ, J., AND DONATIEN, A.: Anns. Inst. Pasteur, 1925, **39**, 925.

Salivary gland disease of guinea pigs

(300) COLE, R., AND KUTTNER, A. G.: Jour. Exp. Med., 1926, **44**, 855.

Measles and German measles

(301) ANDERSON, J. F., AND GOLDBERGER, J.: Pub. Health Rep., 1911, **26**, 847, 887.
(302) BLAKE, F. G., AND TRASK, J. D.: Jour. Exp. Med., 1921, **33**, 385.
(303) CARONIA, G.: La Pediatria, 1923, **31**, 801.
(304) CENCI, F.: Rivista Clin. Pediat., 1907, **5**, 1017.
(305) DEBRÉ, R., JOANNON, P., AND PAPP, K.: Anns. Méd., 1926, **20**, 343.
(306) DEGKWITZ, R.: Münch. med. Woch., 1926, **73**, 181, 248.
(307) DONGES: Centr. Bak., Abt. 1, Orig., 1923, **91**, 45; 1925, **94**, 115.
(308) DUVAL, C. W., AND D'AUNOY, R.: Jour. Exp. Med., 1922, **35**, 257.
(309) FERRY, N. S., AND FISHER, L. W.: Jour. Amer. Med. Assoc., 1926, **86**, 932.
(310) GALLI, P.: La Pediatria, 1922, **30**, 898.
(311) DE GRÖER, Fr., AND REDLICH, Fr.: Compt. rend. Soc. biol., 1926, **94**, 354; Ergeb. inner. Med. u. Kinderheilk., 1926, **30**, 506.
(312) HECHT, A. Fr.: Zt. Kinderheilk., 1925, **40**, 309.
(313) HAGEN, W.: Deut. med. Woch., **52**, 871.
(314) HEKTOEN, L.: Jour. Infect. Dis., 1905, **2**, 238.
(315) HIBBARD, R. J., AND DUVAL, C. W.: Proc. Soc. Exp. Biol. and Med., 1926, **23**, 853.
(316) HOYNE, A. L., AND GASUL, B. M.: Jour. Amer. Assoc., 1926, **87**, 1185.
(317) JURGELUNAS, A.: Centr. Bak., Abt. 1, Orig., 1914, **72**, 483.
(318) KAUPE, W.: Med. Klin., 1926, **22**, 213.
(319) KOCHMANN, R.: Deut. Med. Woch., 1926, **52**, 565.
(320) KRAUS, R.: Seuchenbekämpfung, 1926, **3**, 169.
(321) KUSAMA, S., YOKOYAMA, T., AND ITO, N.: Japan Med. World, 1925, **5**, 1.
(322) KUSAMA, S.: Japan Med. World, 1925, **5**, 309.
(323) MEYER, S.: Monatsch. Kinderheilk., 1926, **31**, 270.
(324) NASSO, I., AND LAURINSICH, A.: Zt. Kinderheilk., 1925, **39**, 516.
(325) NEVIN, M., AND BITTMAN, F. R.: Jour. Infect. Dis., 1921, **29**, 429.
(326) NOBEL, E., AND SCHÖNBERGER, M.: Zt. Kinderheilk., 1925, **39**, 434; **40**, 197.
(327) NOBEL, E.: Monatsch. Kinderheilk., 1926, **31**, 275.
(328) NOEGGERATH, C., OSTER, H., AND VIETHEN, A.: Klin. Woch., 1926, **5**, 1168.
(329) PURDY, W. J.: Brit. Jour. Exp. Path., 1925, **6**, 210.

(330) REUTER, A.: Med. Klin., 1926, **22**, 1181.

(331) SALIMBENI, A.-T., AND KERMORGANT, Y.: Compt. rend. Acad. Sc., 1923, **177**, 717.

(332) SALISBURY, J. H.: Amer. Jour. Med. Sc., 1862, n.s. **44**, 387.

(333) SCHILLING, E.: Klin. Woch., 1922, **1**, 2471.

(334) SELLARDS, A. W., AND BIGELOW, G. H.: Jour. Med. Research, 1920–21, **42**, 241.

(335) SELLARDS, A. W.: Medicine, 1924 **3**, 99.

(336) SCOTT, J. M., AND SIMON, C. E.: Amer. Jour. Hyg., 1924, **4**, 559, 725.

(337) SMOIRA, J.: Zt. Kinderheilk., 1925, **40**, 425.

(338) TAKAKI, J.: Wien. klin. Woch., 1926, **39**, 325.

(339) TUNNICLIFF, R.: Jour. Amer. Med. Assoc., 1917, **68**, 1028; 1918, **71**, 104; Jour. Infect. Dis., 1918, **22**, 462; 1919, **24**, 76, 181; 1925, **37**, 193.

(340) TUNNICLIFF, R., AND BROWN, M. W.: Jour. Infect. Dis., 1918, **23**, 572.

(341) TUNNICLIFF, R., AND MOODY, W. B.: Jour. Infect. Dis., 1922, **31**, 382.

(342) TUNNICLIFF, R., AND HOYNE, A. L.: Jour. Infect. Dis., 1926, **38**, 48.

(343) TUNNICLIFF, R., AND TAYLOR, R. E.: Jour. Amer. Med. Assoc., 1926, **87**, 846.

(344) WIESE, O.: Münch. med. Woch., 1926, **73**, 184.

Grippe (influenza)

(345) DUJARRIC DE LA RIVIÈRE, R.: Compt. rend. Acad. Sc., 1918, **167**, 606.

(346) McINTOSH, J.: Medical Research Council, Special Report Series, No. 63, London, 1922.

(347) NICOLLE, C., AND LEBAILLY, C.: Compt. rend. Acad. Sc., 1918, **167**, 607; Anns. Inst. Pasteur, 1919, **33**, 395.

(348) OLITSKY, P. K., AND GATES, F. L.: Jour. Amer. Med. Assoc., 1920, **74**, 1497; Jour. Exp. Med., 1921, **33**, 125, 361; 1922, **36**, 501; Science, 1923, **57**, 159.

(349) YAMANOUCHI, T., SAKAKAMI, K., AND IWASHIMA, S.: Lancet, 1919, **1**, 971; Compt. rend. Acad. Sc., 1919, **168**, 1347.

(350) ZINSSER, H.: Medicine, 1922, **1**, 213.

Common colds

(351) FOSTER, G. B., JR.: Jour. Amer. Med. Assoc., 1916, **66**, 1180; Jour. Infect. Dis., 1917, **21**, 451.

(352) KRUSE, W.: Münch. med. Woch., 1914, **61**, 1547.

(353) OLITSKY, P. K., AND McCARTNEY, J. E.: Jour. Exp. Med., 1923, **38**, 427.

Nairobi disease of sheep

(354) MONTGOMERY, E.: Jour. Comp. Path. and Therap., 1917, **30**, 28.

Catarrhal fever of sheep

(355) HUTYRA, F., AND MAREK, J.: Pathologie and Therapie der Haustiere, 4th ed., Jena, 1913, vol. i, p. 420.

(356) SPREULL, J.: Jour. Comp. Path. and Therap., 1905, **8**, 321.

African horse sickness

(357) EDINGTON, A.: Jour. Comp. Path. and Therap., 1900, **13**, 200, 281.
(358) KUHN, P.: Centr. Bak., Beilage, Abt. 1, Ref., 1911, **50**, *31.
(359) M'FADYEAN, J.: Jour. Comp. Path. and Therap., 1900, **13**, 1; 1901, **14**, 103.
(360) THEILER, A.: Union of South Africa, Dept. Agric. Science Bull., No. 19, Pretoria, 1921 (Abst. Trop. Vet. Bull., 1922, **10**, 44).

Pappataci fever

(361) DOERR, R.: Berl. klin. Woch., 1908, **45**, 1847.
(362) DA ROCHA-LIMA, H.: v. Prowazek, Handbuch der pathogenen Protozoen, 1920, Leipzig, **2**, p. 985.

Dengue fever

(363) ASHBURN, P. M., AND CRAIG, C. F.: Jour. Infect. Dis., 1907, **4**, 440.
(364) DA ROCHA-LIMA, H.: v. Prowazek, Handbuch der pathogenen Protozoen, 1920, Leipzig, **2**, p. 986.

Yellow fever

(365) NOGUCHI, H.: Jour. Exp. Med., 1919, **29**, 547, 565, 585; **30**, 1, 9, 13, 87, 95, 401; 1920, **31**, 135, 159.
(366) REED, W., AND CARROLL, J.: Jour. Exp. Med., 1900, **5**, 215; American Medicine, 1902, February 22.
(367) REED, W., AND OTHERS: Yellow Fever. A compilation of various publications. U. S. Senate, 1911. Document No. 822.
(368) DA ROCHA-LIMA, H.: v. Prowazek, Handbuch der pathogenen Protozoen, 1920, Leipzig, **2**, p. 980.

Rickettsiae and disease

(369) COWDRY, E. V.: Arch. Path. and Lab. Med., 1926, **2**, 59.
(370) WOLBACH, S. B.: Jour. Amer. Med. Assoc., 1925, **84**, 723.

Typhus fever

(371) HACH, I. W.: Zt. Hyg., 1926, **106**, 221.
(372) KRONTOWSKI, A. A., AND HACH, I. W.: Münch. med. Woch., 1923, **70**, 144; Klin. Woch., 1924, **3**, 1625.
(373) KUCZYNSKI, M. H.: Med. Klin., 1920, **16**, 706, 733, 759; Berlin. klin. Woch., 1921, **58**, 1489; Virch. Arch., 1923, **242**, 355.
(374) KUCZYNSKI, M. H., AND BRANDT, E.: Krankheitsforschung, 1925, **2**, 70.
(375) NICOLLE, C., CONOR, A., CONSEIL, E.: Anns. Inst. Pasteur, 1911, **25**, 97.
(376) OLITSKY, P. K.: Jour. Exp. Med., 1921, **34**, 365, 525; 1922, **35**, 115, 121, 469; 1923, **38**, 691; 1924, **39**, 331; Jour. Amer. Med. Assoc., 1922, **78**, 571.
(377) OTTO, R., AND WINKLER, F.: Zt. Hyg., 1921, **93**, 1.
(378) DA ROCHA-LIMA, H.: v. Prowazek, Handbuch der pathogenen Protozoen, 1920, Leipzig, **2**, p. 990.
(379) WOLBACH, S. B., AND SCHLESINGER, M. J.: Jour. Med. Research, 1923-1924, **44**, 231.

Trench fever

(380) SWIFT, H. F.: Harvey Lectures, 1919–1920, p. 58.
(381) DA ROCHA-LIMA, H.: v. Prowazek, Handbuch der pathogenen Protosoen, 1920, Leipzig, 2, p. 1031.

Rocky Mountain spotted fever

(382) NOGUCHI, H.: Jour. Exp. Med., 1923, 37, 383; 38, 605; 1926, 43, 515.
(383) RICKETTS, H. T.: Jour. Infect. Dis., 1907, 4, 141.
(384) RICKETTS, H. T., AND GOMEZ, L.: Jour. Infect. Dis., 1908, 5, 221.
(385) SPENCER, R. R., AND PARKER, R. R.: Public Health Rep., 1924, 39, 3027; 1925, 40, 2159; 1926, 41, 461, 1817.
(386) WOLBACH, S. B., AND SCHLESINGER, M. J.: Jour. Med. Research, 1923–1924, 44, 231.
(387) DA ROCHA-LIMA, H.: v. Prowazek, Handbuch der pathogenen Protosoen, 1920, Leipzig, 2, p. 1027.

Heartwater disease

(388) COWDRY, E. V.: Jour. Exp. Med., 1925, 42, 231, 253.

Flood fever of Japan

(389) KAWAMURA, R.: Studies on Tsutsugamushi Disease (Japanese Flood Fever), 1926. Special nos. 1 and 2 of vol. iv, Med. Bull., College of Med., University of Cincinnati.

Oroya fever and verruga peruviana

(390) NOGUCHI, H., AND BATTISTINI, T. S.: Jour. Exp. Med., 1926, 43, 851.
(391) NOGUCHI, H.: Jour. Exp. Med., 1926, 44, 533; 1927, 45, 175.
(392) DA ROCHA-LIMA, H.: v. Prowazek, Handbuch der pathogenen Protosoen, 1920, Leipzig, 2, p. 973.

Pleuropneumonia

(393) BORDET, J.: Anns. Inst. Pasteur, 1910, 24, 161.
(394) BORREL, DUJARDIN-BEAUMETZ, JEANTET, AND JOUAN: Anns. Inst. Pasteur, 1910, 24, 168.
(395) NOCARD AND ROUX: Anns. Inst. Pasteur, 1898, 12, 240.

Avian diphtheria

(396) BORDET, J.: Bull. Soc. Roy. Sc. méd. et. nat., Bruxelles, 1907, 65, 125; 1909, 67, 196; Centr. Bak., Abt. 1, Orig., 1913, 67, 41.
(397) BORDET, J., AND FALLY, V.: Anns. Inst. Pasteur, 1910, 24, 563.

Scarlet fever

(398) BROKMAN, H., AND OTHERS: Compt. rend. Soc. biol., 1925, 93, 944.
(399) CARONIA, G., AND SINDONI, M. B.: La Pediatria, 1923, 31, No. 14.

(400) Di Cristina and Caronia: Pediatria Archivio, 1925, **1, 3**.

(401) Dick, G. F., and Dick, G. H.: Jour. Amer. Med. Assoc., 1923, **81**, 1166; 1924, **82**, 265, 301, 544.

(402) Dochez, A. R., and Sherman, L.: Jour. Amer. Med. Assoc., 1924, **82**, 542.

(403) Gabritschewsky, G.: Centr. Bak., Abt. 1, Orig., 1906, **41**, 719 and 844.

(404) Mair, W.: Lancet, 1923, **2**, 1390.

(405) Moser, P.: Wien. klin. Woch., 1902, **15**, 1053.

(406) Nicolle, C., and others: Arch. Inst. Pasteur, Tunis, 1926, **15**, 229.

(407) Park, W. H.: Jour. Amer. Med. Assoc., 1925, **85**, 1180.

(408) Schultz, W., and Charlton, W.: Zt. Kinderheilk., 1918, **17**, 328.

(409) Zlatogoroff, S. J., and others: Centr. Bak., Abt. 1, Orig., 1926, **97**, 152.

(410) da Rocha-Lima, H.: v. Prowazek, Handbuch der pathogenen Protozoen, 1920, Leipzig, **2**, p. 951.

PLATE 1

Figs. 1 and 2.   Hyperplasia and necrosis in mosaic tomato fruits.   Compare with figures 3 and 4.   (Reproduced from Jour. Agric. Research, 1925, 30, 871, by the courtesy of M. W. Gardner.)

Figs. 3 and 4.   Stimulation and destruction of epidermal cells by the virus of chicken-pox.   Compare with figures 1 and 2.

Fig. 5. Section of rabbit's cornea removed forty-eight hours after inoculation with herpes virus.   Intracellular changes, nuclear inclusions, are only in the young cells filling the defect caused by scarification.

FURTHER INVESTIGATIONS OF THE RELATION BE-TWEEN THE CHEMICAL CONSTITUTION AND THE GERMICIDAL ACTIVITY OF ALCOHOLS AND PHENOLS

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The relation between the chemical constitution and the germicidal activity of certain monohydric alcohols and phenols has already been discussed (Tilley and Schaffer, 1926). The present paper will report an extension of these studies to other monohydric alcohols and phenols and to some dihydric phenols and the corresponding intermediate ketones.

MATERIALS

Some of the compounds studied were secured from commercial sources; others were obtained through the courtesy of Dr. Veader Leonard, of Johns Hopkins University, to whom we are greatly indebted while still others were prepared in this laboratory. Previous work having demonstrated the necessity for a very high degree of purity, considerable attention was devoted to the adequate purification of the samples employed. In the case of the compounds obtained from commercial sources, efforts were made to ascertain the methods of production. Steps were then taken to purify each compound by the application of appropriate methods of fractional distillation, crystallization, or extraction. The compounds obtained in this manner were phenol, resorcinol, orcinol, the cresols, the cyclohexanols, the xylenols, the alcohols, thymol, and carvacrol. The substances placed at our disposal by Leonard comprised the n-alkyl resorcinols from n-propyl resorcinol to n-octyl resorcinol inclusive, the isoresorcinols from

isobutyl resorcinol to isohexyl resorcinol inclusive, and the corresponding intermediate ketones of both series. Dohme (Sharpe and Dohme and Dohme, 1925) has reported melting points for some of these compounds and as the preparations furnished us by Leonard were found to melt at the corresponding temperatures and as they appeared to be already of a high degree of purity, they were at first used in the bacteriological tests without further attempts at purification. In tables 1 and 5 these preparations are indicated by the addition of "A_1" to the names of the respective compounds.

In testing the bacteriological action of these preparations, however, we were not always able to duplicate the results of previous investigators (Johnson and Lane, 1921; Leonard, 1924), and since we noted marked irregularities in our own results we decided to attempt the further purification of the samples of the n-alkyl resorcinols received from Leonard. The members of the n-alkyl resorcinol series were therefore recrystallized from various solvents, for the most part from petroleum ether and from mixtures of petroleum ether and benzene, until by repetition of the process no further change could be produced in the melting points and bactericidal properties. It appears that in this way certain impurities of high bactericidal power were eliminated from the samples of n-butyl resorcinol and n-amyl resorcinol, but no appreciable change was produced in the other n-alkyl resorcinols. The recrystallized resorcinols have been designated by the symbol "A_2" in tables 1 and 5.

In view of the variations in bactericidal properties shown by some of these compounds in the course of several recrystallizations, it was thought best to check these results by carrying out another series of tests with materials obtained from another source. Accordingly, the following compounds were prepared in this laboratory—n-propyl resorcinol, n-butyl resorcinol, n-amyl resorcinol, and n-octyl resorcinol; and at the same time the series was extended to include ethyl resorcinol, n-nonylresorcinol, and a mixture consisting largely of n-octyl and n-decyl resorcinols. The method used in the preparation of these compounds was that reported by Johnson and Lane (1921), the fatty acids employed

being very highly purified except in the case of the mixture mentioned above.   The ethyl resorcinol, n-propyl resorcinol, n-butyl resorcinol, n-amyl resorcinol, n-octyl resorcinol, and n-nonylresorcinol were recrystallized until no further change was produced in the melting point or bactericidal properties, the finally purified samples being designated by "B₂" in tables 1 and 5.   In the case of n-butyl resorcinol and n-amyl resorcinol, partially purified products were also examined and have been designated as "B₁"¹ In the preparation of the mixture of alkyl resorcinols, the fatty acid used was a mixture obtained by the fractional distillation of the fatty acids of coconut oil and had an average molecular weight of 168 and a boiling point of 262°–285°.   In view of the analysis of coconut oil reported by Armstrong et al. (1925), the sample of mixed alkyl resorcinols was presumably a mixture of n-octyl and n-decyl resorcinols together with small amounts of higher homologues.   For the sake of convenience this mixture of resorcinols has been designated "coconut" resorcinol.

In addition to the compounds already mentioned, some of the para-alkyl phenols were also prepared.   In the preparation of the p-n-amyl phenol and the p-n-hexyl phenol, the respective fatty acids were first of all combined with anisole in the presence of aluminum chloride to produce the para-alkyl ketone phenyl ether, after which the ketone was reduced with zinc and hydrochloric acid to give the para-alkyl phenyl ether, which in turn was reduced with phosphorus and hydriodic acid to produce the desired phenol.

The p-isoamyl phenol was made by condensing isoamyl alcohol with phenol in the presence of zinc chloride.   While its composition is not definitely known, it probably is either p-isoamyl phenol or p-tertiary amyl phenol.   Para-benzyl phenol was prepared through the action of benzyl chloride on phenol in the presence of zinc.   (Zincke and Walter, 1904).

The constants of the materials as finally used in the bactericidal tests have been assembled in table 1.   For the boiling points,

¹ After the major portion of this work had been completed Dohme et al. (1926) published new constants for some of the alkyl resorcinols originally mentioned by Dohme and in addition added constants for those alkyl resorcinols named above and not previously reported by Dohme.

TABLE 1

Physical constants of the substances tested

SUBSTANCE	FORMULA	BOILING POINT	B.P.°
Phenol	C_6H_5OH	181.7	40.7
Para-n-amyl phenol	$C_5H_{11}\cdot C_6H_4OH$	262.5–267.5	
Para-n-hexyl phenol	$C_6H_{13}\cdot C_6H_4OH$	280	29.85
Ortho-cresol	$CH_3\cdot C_6H_4OH$	190.8 –191.1	
Meta-cresol	$CH_3\cdot C_6H_4OH$	202.17–202.22	
Cyclohexanol	$(CH_2)_5{:}CHOH$	160.2 –161.5	
Ortho-methylcyclohexanol	$CH_3CH{:}(CH_2)_4{:}CHOH$	165.5 –167.5	
Meta-methylcyclohexanol	$CH_3CH{:}(CH_2)_4{:}CHOH$	173.2 –174.6	
Para-methylcyclohexanol	$CH_3CH{:}(CH_2)_4{:}CHOH$	172.7 –174.7	
Vicinal meta-xylenol (2-6-dimethyl phenol)	$(CH_3)_2\cdot C_6H_3OH$	201	45.5
Para-xylenol (2-5-dimethyl phenol)	$(CH_3)_2\cdot C_6H_3OH$		75
Asymmetric meta-xylenol (2-4-dimethyl phenol)	$(CH_3)_2\cdot C_6H_3OH$	211.3 –211.7	19.5
Asymmetric ortho-xylenol (3-4-dimethyl phenol)	$(C'H_3)_2\cdot C_6H_3OH$	225.8 –226.1	64.2
Thymol (3-methyl-6-isopropyl phenol)	$(CH_3)_2CH\cdot C_6H_3\cdot CH_3\cdot OH$		49.35
Carvacrol (2-methyl-5 isopropyl phenol)	$(CH_3)_2CH\cdot C_6H_3\cdot CH_3\cdot OH$	237.5 –238.5	
Para-benzyl phenol	$C_6H_5\cdot CH_2C_6H_4OH$		83
Para iso? amyl phenol	$C_5H_{11}\cdot C_6H_4OH$	253–260	89
Diethyl carbinol	$(C_2H_5)_2{:}CHOH$	115–118	
Triethyl carbinol	$(C_2H_5)_3{:}COH$	141–143	
Benzyl alcohol	$C_6H_5\cdot CH_2OH$	206–206.5	M.P.°
Phenyl ethyl alcohol	$C_6H_5\cdot CH_2CH_2OH$	219–219.5	
Resorcinol (1-3-hydroxybenzene)	$C_6H_4(OH)_2$		110
Orcinol (1-3-hydroxy-5-methyl benzene)	$CH_3\cdot C_6H_3(OH)_2$		107–108
Ethyl resorcinol (B₂) (1-3-hydroxy-4-ethyl benzene)	$C_2H_5\cdot C_6H_3(OH)_2$		97–98

		B.P.	M.P.
n-Propyl resorcinol (A₇–B₂)	$C_3H_7 \cdot C_6H_3(OH)_2$		79.2–79.4
n-Butyl resorcinol (A₇–B₂)	$C_4H_9 \cdot C_6H_3(OH)_2$		51.4–51.7
n-Butyl resorcinol (A₁)			48.5–49.5
n-Butyl resorcinol (B₁)			49 –49.5
n-Amyl resorcinol (A₇–B₂)	$C_5H_{11} \cdot C_6H_3(OH)_2$		72–73
n-Amyl resorcinol (A₁)			69–70
n-Amyl resorcinol (B₁)			72–73
n-Hexyl resorcinol (A₂)	$C_6H_{13} \cdot C_6H_3(OH)_2$		62 –62.5
n-Heptyl resorcinol (A₂)	$C_7H_{15} \cdot C_6H_3(OH)_2$	200/9 mm.	70.4–70.8
n-Octyl resorcinol (A₇–B₂)	$C_8H_{17} \cdot C_6H_3, OH)_2$	198–199/3 mm.	70.2–70.6
n-Nonyl resorcinol (B₁)	$C_9H_{19} \cdot C_6H_3(OH)_2$		72.5–73
Coconut resorcinol	$C_{10}H_{21} \cdot C_6H_3(OH)_2$	200–225/4 mm.	60–62
Isobutyl resorcinol (A₁)	$(CH_3)_2CH \cdot CH_2 \cdot C_6H_3(OH)_2$		61–62
Isoamyl resorcinol (A₁)	$(CH_3)_2CH \cdot (CH_2)_2 \cdot C_6H_3(OH)_2$		59–60
Isohexyl resorcinol (A₁)	$(CH_3)_2CH \cdot (CH_2)_3 \cdot C_6H_3(OH)_2$		69.5–70
Acetyl resorcinol (A₁)	$CH_3CO \cdot C_6H_3(OH)_2$		143.5–144.5
Propionyl resorcinol (A₁)	$CH_3CH_2CO \cdot C_6H_3(OH)_2$		95.5–96.5
Butyryl resorcinol (A₁)	$CH_3(CH_2)_2CO \cdot C_6H_3(OH)_2$		69 –69.5
n-Amylyl resorcinol (A₁)	$CH_3(CH_2)_3CO \cdot C_6H_3(OH)_2$		55 – 55.5
n-Hexylyl resorcinol (A₁)	$CH_3(CH_2)_4CO \cdot C_6H_3(OH)_2$		55 – 55.5
n-Heptylyl resorcinol (A₁)	$CH_3(CH_2)_5CO \cdot C_6H_3(OH)_2$		50 – 51
n-Octylyl resorcinol (A₁)	$CH_3(CH_2)_6CO \cdot C_6H_3(OH)_2$		58.5– 59.5

* S.P. = solidification point.   M.P. = melting point.

standardized Anschutz thermometers were used, the stems being immersed in the vapors of the liquid during distillation. When the determinations were made at prevailing atmospheric pressures, which varied between 755 and 775 mm., the observed temperatures were corrected to normal pressure by use of the table of Young (1902). The solidification points were determined, when a sufficient quantity of material was at hand, by immersion of the bulb and part of the stem of the thermometer in the material as it solidified. The melting points were determined by the capillary tube method in the usual manner.

BACTERIOLOGICAL WORK

For the purposes of the present investigation, just as in our previous work, the germicidal efficiency of the various compounds studied has been measured by the phenol coefficients determined by a modified Rideal-Walker method. The exact technic has been described in our previous paper but we wish again to emphasize the fact that the coefficients obtained by this method, unlike those determined by other methods, are based on the relative amounts, by weight, of the disinfectant and of the phenol standard required to kill the test organism in the same length of time.

In addition to the ordinary phenol coefficients there are also shown in the tables what are designated as "molecular" coefficients. As explained in our previous paper, these are the phenol coefficients reduced from their original gram-weight basis to a gram-molecular basis.

In direct continuation of our previous work with the para phenols, p-n-amyl phenol and p-n-hexyl phenol were prepared and tested. The results obtained are shown in table 2 with results reported in the previous paper, which are repeated for convenient reference.

In the column headed "Ratio" there are shown the ratios between the molecular coefficients of successive members of the series. It will be seen that the average ratio of 3.3 mentioned in our previous paper holds true with *B. typhosus* as the test organism up to the coefficient of p-n-hexyl phenol, which is less than the figure expected. In this respect it resembles the coef-

ficients of the higher resorcinols, as will be seen later. The average ratio with *Staph. aureus* as the test organism is 3.1 for the entire series.

The molecular coefficients of the higher members of the series have been calculated from the coefficients of para cresol by use of these ratios and the coefficients thus obtained are shown in table 2 under the heading "Calculated." It will be seen that they correspond very closely to the observed coefficients, except in the case of p-n-hexyl phenol with *B. typhosus* as the test organism.

A number of additional phenols and alcohols were also examined. The results obtained are shown in table 3.

TABLE 2

Coefficients of the para-phenols

NAME	TEST ORGANISM B. TYPHOSUS				TEST ORGANISM STAPH. AUREUS			
	Phenol coefficient	Molecular coefficients			Phenol coefficient	Molecular coefficients		
		Observed	Ratio	Calculated		Observed	Ratio	Calculated
Cresol..................	2.5	2.9	3.3		2.2	2.5	3.1	
Ethyl phenol...........	7.4	9.6	3.2	9 6	6	7.8	3.1	7.8
Propyl phenol..........	21.6	31	3.5	31.7	16.5	24	3.3	24
Butyl phenol..........	68	108	3.2	105	50	79	3	75
Amyl phenol...........	197	344	2.7	346	139	243	2.9	232
Hexyl phenol...:.......	500	950		1,140	375	710		720

Examination of the results shown in tables 2 and 3 indicates that the germicidal activity of isomeric compounds may vary widely, depending on the relative positions of the various constituent groups. Examples of this may be seen by comparing the coefficients of p-cresol and p-ethyl phenol with those of benzyl alcohol and phenylethyl alcohol, respectively, or the coefficient of p-ethyl phenol with the coefficients of the xylenols, or the coefficients of n-butyl phenol with the coefficients of thymol and carvacrol. So far as the isomeric phenols are concerned it appears that maximum efficiency is shown by those in which normal alkyl chains are attached to the benzene ring in a position para to the hydroxyl group, and that among these para

compounds the bactericidal power increases with the increase in length of the alkyl chains.

If the results here reported for diethyl and triethyl carbinols be considered with the results for other alcohols reported in our previous paper it seems evident that among isomeric alcohols greatest efficiency is shown by compounds having the longest straight chains.

TABLE 3

Phenol coefficients of certain monohydric alcohols and phenols

SUBSTANCES	PHENOL COEFFICIENT	MOLECULAR COEFFICIENT
o-Cresol	2.2	2.5
m-Cresol	2.4	2.8
Cyclohexanol	0.52	0.55
o-Methyl cyclohexanol	1.05	1.27
m-Methyl cyclohexanol	1.25	1.52
p-Methyl cyclohexanol	1.30	1.58
p-Xylenol	5.5	7.1
Asymmetric o-xylenol	5.3	6.9
Asymmetric m-xylenol	5.8	7.5
Vicinal m-xylenol	4.2	5.5
Thymol	28.5	45
Carvacrol	27.5	44
p-Benzyl phenol	62	121
p-Isoamyl phenol	74	129
Diethyl carbinol	0.36	0.34
Triethyl carbinol	0.95	1.17
Benzyl alcohol	0.76	0.87
Phenyl ethyl alcohol	0.90	1.17

Test organism, *B. typhosus*, strain no. 1.

It is interesting to note that the coefficients of cyclohexanol and the methyl cyclohexanols are approximately half as great as those of phenol and the cresols, while the relative efficiency of the isomers remains in the same order of para, meta, and ortho.

In table 4 there are shown the results obtained with the normal and isoresorcinols and the normal ketones, except the acetyl and octylyl resorcinols. No results are shown for these two ketones because in each case a saturated solution failed to kill *B. typhosus* within the time limits of the test. The isobutyl, isoamyl, and

isohexyl ketones also were so little soluble as to prevent obtaining coefficients for them.

Orcinol should properly not be included in this homologous series of resorcinols because its methyl group is meta to both hydroxyl groups, whereas its isomer of this series would have its methyl group para to one hydroxyl group and ortho to the other. It

TABLE 4

Coefficients of the normal and iso-resorcinols and normal ketones

	PHENOL COEFFI- CIENTS	MOLECULAR COEFFICIENTS		
		Observed	Ratio	Calculated
Normal resorcinols:				
Resorcinol...............................	0.37			
Orcinol................................	0.53			
Ethyl resorcinol........................	1.6	2.35	3.32	
Propyl resorcinol.......................	4.8	7.8	3.39	7.9
Butyl resorcinol........................	15	26.5	3.39	26.6
Amyl resorcinol........................	47	90	3.37	89.6
Hexyl resorcinol.......................	147	303	2.53	302
Heptyl resorcinol......................	350	770	1.22	1,020
Octyl resorcinol........................	400	940		3,400
Coconut resorcinol..........	100			
Isoresorcinols:				
Isobutyl resorcinol.....................	12.8	22.6	3.8	
Isoamyl resorcinol.....................	46	88	2.4	70
Isohexyl resorcinol....................	105	217		217
Normal ketones:				
Butylyl resorcinol......................	13.5	26	3	
Amylyl resorcinol.....................	37.5	78	3	77
Hexylyl resorcinol.....................	106	234	2.8	232
Heptylyl resorcinol....................	280	660		700

Test organism, *B. typhosus*, strain no. 1.

seemed likely, however, that the coefficient of orcinol would be near enough to that of its isomer to indicate what the latter might be and it was therefore included in the table.

In the case of the n-butyl and n-amyl resorcinols, purification had a marked effect on germicidal efficiency, as will be seen from the results shown in table 5. The samples marked A₁ and B₁

were tested before complete purification and those marked A₂ and B₂ after complete purification.

In connection with the molecular coefficients there are shown in table 4 in the column headed "Ratio," the ratios between the coefficients of successive members of each series. Although there are irregularities it seems that with *B. typhosus* as a test organism the average ratio for the normal resorcinols from ethyl to hexyl, inclusive, is 3.37 while the ratios for the isoresorcinols and normal ketones are approximately 3.1 and 3.0, respectively.

The coefficients of the higher members of each series have been calculated from the coefficient of the lowest member by the use of these ratios and the coefficients thus obtained are shown in table 4 in the column headed "Calculated." In the normal

TABLE 5

Phenol coefficients of resorcinols before and after purification

	COEFFICIENT		COEFFICIENT
N-Butyl—A₁*................	21	N-Amyl—A₁................	51
N-Butyl—A₂................	15	N-Amyl—A₂................	47
N-Butyl—B₁................	26	N-Amyl—B₁................	53
N-Butyl—B₂................	15	N-Amyl—B₂................	47

Test organism, *B. typhosus*, strain no. 1.

* A₁, B₁, before purification; A₂, B₂, after purification.

resorcinol series the observed and calculated coefficients correspond very closely up to that of n-heptyl resorcinol, where there is a decided difference which is still more marked in the case of n-octyl resorcinol. The same sort of difference between observed and calculated coefficients is seen in the case of the n-heptyl ketone. With "coconut" resorcinol the difference between the observed and the estimated coefficient is even more marked than with n-octyl resorcinol.

When the higher resorcinols were tested at higher temperatures and over longer periods of time there was an increase in germicidal efficiency which was most marked in the highest members of the series. With n-hexyl resorcinol a dilution of 1:16000 killed *B. typhosus* in ten minutes at 25°, and 1:20,000 in thirty minutes

at 37.5,° while with n-octyl resorcinol a dilution of 1:10,000 killed the organism in twelve minutes at 20°, 1:40,000 in ten minutes at 25°, 1:120,000 in ten minutes at 37.5°, 1:100,000 in thirty minutes at 25°, and 1:160,000 in forty-five minutes at 37.5°. In a similar manner *B. typhosus* was killed by "coconut" resorcinol in ten minutes at a dilution of 1:10,000 at 25° and at a dilution of 1:40,000 at 37.5°, and by n-heptyl resorcinol at a dilution of 1:35,000 in ten minutes at 25°, and 1:60,000 in thirty minutes at 37.5°.

TABLE 6

Phenol coefficients of phenols and alcohols with different strains of B. typhosus

	STRAIN NO. 1	STRAIN NO. 2	STRAIN NO. 3	STRAIN NO. 4	STRAIN NO. 5
Alcohols:					
Primary amyl...............	0.78	0.74	0.75	0.76	0.78
Primary hexyl..............	2.3	2.25	2.25	2.25	2.25
Primary heptyl.............	6.8	6.5	6.5	6.8	6.6
Primary octyl...............	21	20	20	20	20
Para phenols:					
Cresol.......................		2.35	2.45	2.4	2.35
Ethyl phenol................		6.6	6.8	6.6	6.3
Propyl phenol...............	19	18	18	18	18
Butyl phenol................	65	63	64	66	64
Amyl phenol................	197	42	45	74	55
Hexyl phenol...............	500	*	*	*	*

* No coefficient obtainable. A saturated solution failed to kill the test organism in fifteen minutes.

The coefficients obtained for the higher resorcinols as shown in table 4 are much greater than the coefficients reported previously by other investigators. In attempting to ascertain the cause of this discrepancy we found that the strain of *B. typhosus* employed throughout our work up to that time, and which will be designated hereafter as strain no. 1, was much more sensitive to the higher resorcinols than other strains which were available, while the resistance of all the strains to phenol was approximately the same.

These results suggested two possiblities: first, that strain no. 1 was really not *B. typhosus* at all, and second, that the ratios

previously noted and reported might be true only for that particular strain. Strain no. 1 therefore, was, carefully compared with other strains of *B. typhosus* which were available and all were positively identified as *B. typhosus*. Comparatve tests were then made with these strains of *B. typhosus* on the paraphenols

TABLE 7

Phenol coefficients of the normal resorcinols with different strains of B. typhosus

NAME	STRAIN NO. 1	STRAIN NO. 2	STRAIN NO. 3	STRAIN NO. 4	STRAIN NO. 5
Propyl.	4.8	4.75	4.76	4.76	4.75
Butyl.	15	14.6	15.3	15.6	14.6
Amyl.	47	40	40	53	48
Hexyl.	147	52	58	65	61
Heptyl.	350	31	34	42	34
Octyl.	400	*	*	*	*
Nonyl.	*				

* No coefficient obtainable.

TABLE 8

Coefficients of the normal resorcinols with different strains of Staphylococcus aureus

NAME	PHENOL COEFFICIENTS			STRAIN NO. 3	
	Strain no. 1	Strain no. 2	Strain no. 3	Molecular coefficients	Ratios
Ethyl.	1.55	1.5	1.5	2.2	2.7
Propyl.	3.9	3.7	3.7	6	3
Butyl.	10.7	10	10.2	18	3.2
Amyl.	30.2	30.2	30.6	59	3.4
Hexyl.	97	98	98	202	3.1
Heptyl.	295	280	280	620	2.7
Octyl.	690	680	725	1,710	1.5
Nonyl.	1,000	980	1,000	2,500	

and the higher primary alcohols discussed in our previous paper. The results of these comparative tests are shown in table 6.

It is evident from table 6 that so far as the alcohols and lower phenols are concerned there is practically no difference in their action on different strains of *B. typhosus*. On the other hand there are very great differences with p-n-amyl phenol and p-n-hexyl phenol.

A similar comparative study was made with the normal resorcinols, the results of which are shown in table 7.   It will be seen that the lower resorcinols, like the lower phenols, yield uniform results with different strains of *B. typhosus* while the higher resorcinols resemble the higher phenols in showing great differences in their action on different strains of *B. typhosus*.

Tests were made on the normal resorcinols with three different strains of *Staph. aureus*, the results of which are shown in table 8.   It will be noted that the phenol coefficients of the various normal resorcinols are practically the same with all three strains of *Staph. aureus*.   For that reason molecular coefficients and ratios are shown for only one strain.   The average ratio for the series from ethyl to heptyl is 3.1, the ratios for the higher members of the series being much lower.   The n-amyl and n-hexyl phenols were also tested with the same three strains of *Staph. aureus* and the results obtained for each of these compounds were very much alike with all three strains.

DISCUSSION

Consideration of the results reported previously, together with those of the present investigation discloses the interesting fact that with *B. typhosus* as the test organism the average ratio of 3.37 for the molecular coefficients of successive members of the normal resorcinol series is very close to the ratios 3.36 and 3.3 for the primary alcohols and para-phenols, respectively, while with *Staph. aureus* as the test organism the para-phenols and normal resorcinols have the same average ratio of 3.1.   This similarity in ratios indicates that the bactericidal power of any member of the different series of compounds studied is affected essentially to the same degree by the introduction of the methyl group.   The irregularities in the action of some of the higher phenols and resorcinols and the decrease in the ratios which occurs with the higher members of each series do not seem to us to detract in any essential way from the significance of the ratios which we have reported here and in our previous paper.

The work with the lower members of each series having demonstrated a relation between chemical constitution and bactericidal

action, it became possible to predict the efficiency of compounds which had not yet been prepared. This led to the preparation of compounds of extremely high bactericidal efficiency, the highest of which had a bactericidal efficiency 1,000 times that of phenol, weight for weight. Another point of considerable interest is the "selective" action of the higher phenols and resorcinols against *Staph. aureus*. shown in its highest degree by the remarkably high coefficients of n-octyl and n-nonyl resorcinols. Although these coefficients are much higher than any previously reported for similar compounds, there is no reason to suppose that the upper limit has been reached. If, as seems quite possible, the higher phenols and resorcinols manifest similar "selective" action against other bacteria, it is only natural to suppose that they may be found to be of practical value in medicine.

It must be remembered, however, that although phenol coefficients are quite capable of indicating with great accuracy the relative germicidal activity of different substances of the same class when tested under rigidly controlled laboratory conditions the results can not be accepted as an accurate measure of the practical value of disinfectants. This can be ascertained only by tests carried out under conditions approximating as nearly as possible those under which the disinfectants are to be used.

SUMMARY

In a previous paper the authors have shown that the bactericidal power of the alcohols and phenols increases so uniformly with the molecular weight that the rate of increase may be expressed by definite numerical ratios. The present paper reports the results of similar work with other alcohols and phenols and with various resorcinols and the corresponding intermediate ketones.

The results here reported for alcohols and phenols confirm the conclusions stated in the previous paper. The combined results of both papers indicate that the germicidal activity of the isomeric alcohols and phenols varies widely, depending on the position of the various constituent groups. Among isomeric alcohols greatest efficiency is shown by compounds having the longest straight chains and among isomeric phenols maximum efficiency is shown

by compounds having the longest straight chains in positions para to the hydroxyl group. The coefficients of cyclohexanol and the methyl cyclohexanols were found to be approximately half as great as those of phenol and the corresponding cresols.

The results here reported for the resorcinols and the normal ketones indicate that with *B. typhosus* as the test organism the ratios between successive molecular coefficients are 3.37 for the normal resorcinols, 3.1 for the isoresorcinols, and 3.0 for the normal ketones.

With *Staph. aureus* as the test organism results reported here and in our previous paper indicate an average ratio of 3.1 between successive molecular coefficients for the para-phenols and results reported in the present paper indicate the same ratio of 3.1 for the coefficients of the normal resorcinols.

The higher phenols and higher resorcinols were found to be relatively much more efficient against *Staph. aureus* than against *B. typhosus*.

REFERENCES

ARMSTRONG, E., ALLEN, JOHN, AND MOORE, CHARLES WATSON 1925 The fatty acid constituents of some natural fats. Jour. Soc. Chem. Ind., **44**, 631–681.

DOHME, A. R. L., COX, EDWARD H., AND MILLER, ELLIS 1926 The preparation of the acyl and alkyl derivatives of resorcinol. Jour. Amer. Chem. Soc., **48**, 1688–1693.

JOHNSON, T. B., AND LANE, F. W. 1921 Preparation of some alkyl derivatives of resorcinol and the relation of their structure to antiseptic properties. Jour. Amer. Chem. Soc., **43**, 348–360.

LEONARD, VEADER 1924 Secretion of bactericidal urine and disinfection of the urinary tract following administration of certain alkyl derivatives of resorcinol. Jour. Amer. Med. Assoc., **83**, 2005–2012.

LIEBMAN, A. 1882 Synthese der Homologen Phenols. Ber. der deutsch. Chem. Gesellschaft, **15**, 150–154.

SHARPE AND DOHME, INC., AND DOHME, A. R. L. 1925 Preparation of alkyl and aryl resorcinols. Abstracts of Chemical Papers, Bureau of Chemical Abstracts, **128**, 541 and 809.

TILLEY, F. W., AND SCHAFFER, J. M. 1926 Relation between the chemical constitution and germicidal activity of the monohydric alcohols and phenols. Jour. Bacteriol., **12**, 303–309.

YOUNG, SIDNEY 1902 Correction of boiling points of liquids from observed to normal pressure. Jour. Chem. Soc., **31**, 777–783.

ZINCKE, T., AND WALTER, W. 1904 Ueber die Einwirkungen von Brom auf. p.-oxydiphenylmethan. Liebigs Annalen der Chemie, **334**, 373.

THE DIMENSIONS OF DIVIDING MICROÖRGANISMS

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The statement is commonly made that after fission of a microörganism the diameter of each of the two daughter cells must be one-half that of the single mother cell from which they arise. The statement is applied to the width or to the length according as longitudinal or transverse division respectively is under consideration.

If organisms presented only a plane surface such a statement would be perfectly correct. Inasmuch as they have three dimensions the conception needs qualifications. On dividing, it is not the observed figure in two dimensions which divides but the total volume of the organism. Assuming that division is into two equal parts and that the density does not vary, the resulting daughter cells will have one-half the volume of the single mother cell.

Microörganisms may be thought of as spheres or right cylinders or rectangular parallelopipeds or approximations to these three types of solids.

The volume of a sphere is equal to the surface area times one-third the radius. The surface area is equal to $4\pi\rho^2$. Then the volume, $V = 4\pi\rho^2 \cdot \frac{1}{3}\rho = \frac{4\pi\rho^3}{3}$.

In this formula the radius is the only variable quantity. The volumes of spheres are therefore proportional to the cubes of of their radii,—or the cubes of their diameters.

When organisms are spherical and the daughter cells after division assume the spherical form, as in the case of the micrococci, the volume of each daughter cell will be one-half that of

the mother cell that is $\frac{4\pi\rho^3}{3} \cdot \frac{1}{2}$. The respective radius (or diameter) of the mother cell to that of each daughter cell will be as the radius (or diameter) of the mother cell to the cube root of one-half the cube of the radius (or diameter) of the mother cell respectively. Thus, if the diameter of the mother cell is 4, the diameter of the daughter cell will be

$$\sqrt[3]{\frac{4^3}{2}} = \sqrt[3]{\frac{64}{2}} = \sqrt[3]{32} = 3.176$$

or over three quarters that of the mother cell. In case of the Gram negative diplococci, or the β hemolytic streptococci the daughter cells are flattened against each other. To the degree that they approximate two hemispheres arising from a sphere, their diameter, perpendicular to the plane of fission, will be the radius of the spherical mother cell, and equal one-half that of the mother cell.

The volume of a right cylinder is equal to the area of the circular base times the altitude. Since the area of a circle is equal to $\pi\rho^2$ the volume of a cylinder is represented by the formula $V = \pi\rho^2 \cdot H$ where V = the volume and H = the altitude. Here there are two variables, the radius and the altitude.

Rod shaped organisms with square ends may be thought of as right cylinders. On transverse division at right angles to the altitude there result two right cylinders whose bases are the same as that of the mother cell, but whose altitude is one-half that of the mother cell. If, however, such a cylinder were to divide longitudinally the dimensions would not necessarily be in such a simple proportion. If the resultant halves of the original cylinder persisted in the form of half cylinders with semicircles as bases, then the diameter of each of these two daughter cells when looked at in the plane of division would present as its diameter the radius, or one-half the diameter of the base of the mother cell. If, however, the two daughter cells became rounded up into two right cylinders and the length or altitude remained unchanged the area of each of their circular bases would be one-half the area of the circular base of the mother cell.

The area of a circle is equal to $\pi\rho^2$ the radius being the only variable. The areas of circles are therefore proportional to the squares of their radii. The base of the mother cell would be to the base of each daughter cell as ρ^2 is to $\sqrt{\frac{\rho^2}{2}}$. The radius (or diameter) of the mother cell will be to the radius (or diameter) of each daughter cell as the radius (or diameter) of the mother cell to the square root of one-half the square of the radius (or diameter) of the mother cell. Thus if the diameter of the mother cell is equal to 4, the diameter of each daughter cell will equal

$$\sqrt{\frac{4^2}{2}} = \sqrt{\frac{16}{2}} = \sqrt{8} = 2.83 -$$

or a little less than three quarters the diameter of the mother cell.

A certain degree of variation from these proportions would be present with variations from the type of the right cylinder. Rounded ends or pointed or tapering ends (in which the terminal portions may be thought of as hemispheres or as right circular cones superimposed upon the right cylinder of the main body of the cell) introduce complicating factors. Whether such factors would be sufficiently large to fall outside of the factor of error where measurements as minute as those of bacteria are under consideration is doubtful. Assuming that the above formulas and proportions hold it is necessary that these be borne in mind when discussing the theoretical possibility of longitudinal division of cylindrical organisms such as for example the spirochetes.

The volume of a rectangular parallelopiped is equal to the product of its three dimensions. Halving of this volume by parallel bisection in any one of the three planes would halve the dimension at right angles to the plane of division. Elongated flattened organisms would fall under this type.

Tapering at the ends or thinning at the edges would introduce other factors. Thus an organism of this general type, but having thin edges so that its cross section would present not a parallelogram' but an ellipse would presumably show a diameter of the daughter cells greater than one-half the diameter of the mother cell if it divided longitudinally through the short axis of the

elliptical cross section so that each edge along the plane of division became thinned out. Such a condition would have to be considered in the case of certain protozoa.

These relations may be objectively illustrated with plastisine models.

SUMMARY AND CONCLUSIONS

Upon equal binary fission a microörganism divides into two daughter cells each having one-half the volume of the mother cell. The diameter of those daughter cells is not always one-half that of the mother cell. In the case of spherical organisms the two resulting spheres will have a diameter equal to the cube root of one-half the cube of the diameter of the mother cell, or almost seven-eighths that of the mother cell. In the case of cylindrical organisms the length of the daughter cells will be one-half that of the mother cell after transverse division. After longitudinal division however, the diameter of the daughter cylinders will be equal to the square root of one-half the square of the diameter of the mother cell, or a little less than three-quarters that of the mother cell. Elongated flat organisms approximating a rectangular parallelopiped will upon division exhibit in the daughter cells a diameter one-half that of the mother cell. Deviations from the type may cause the diameter of the daughter cells to be greater than one-half that of the mother cell.

THE INTERPRETATION OF CHANGES IN ELECTRICAL RESISTANCE ACCOMPANYING THE DEATH OF BACTERIAL CELLS

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Of the four or five methods that have been employed for ascertaining the permeability of living cells to salts, the determination of their electrical resistance is usually regarded as the most direct. This view is based on the assumptions that the passage of ions propelled by a given force into a cell is conditioned by the degree of permeability of the cell membrane, that an electric current is conveyed through an aqueous medium by the bodily migration of ions, and that, in accordance with Ohm's law, $E = CR$, the current flowing between two platinum electrodes connected to a source of constant EMF is inversely proportional to the resistance of the medium between those electrodes. Resistance, or rather its reciprocal, conductivity, is thus said to be a direct measure of the migration of ions, and consequently, also, of the permeability of the cell.

On the basis of these theoretical views Osterhout (1918; 1919; 1920) carried out an extensive series of determinations of the electrical conductivity of various tissues—algal tissues, and one animal tissue, frog skin—when placed in various saline solutions. He concluded from his results that when tissues die, their permeability to ions is increased, that death and increased permeability can be brought about by immersion in solutions of monovalent ions, and that this effect can be antagonised by the addition of divalent ions. He showed moreover, that his results agreed with results obtained by determinations of plasmolysis, tissue tension, and diffusion through membranes of living tissues.

The same method has been applied, and on the same theoretical

assumptions, to suspensions of single cells. A number of investigators have worked with red blood cells, Gray (1916) with echinoderm eggs, and Shearer (1919) with suspensions of bacteria. Their results are neither as constant nor as conclusive as are those of Osterhout with tissues. The earlier workers, among them Stewart (1899; 1909) believed that red blood cells have a remarkably high electrical resistance. Brooks (1925) however, has shown that this view is incorrect. Gray reported an increase in the permeability of eggs at fertilisation, and Shearer claimed to have demonstrated the usual salt antagonisms with suspensions of *Bacillus coli*, and arrived at the conclusion that dead cells offer no resistance to the passage of ions.

The reason why people who worked with suspensions obtained results that were either contradictory or inexplicable, was because they committed the error of adopting unquestioningly and *in toto* the theoretical assumptions that had been shown by Osterhout to be generally applicable to tissues, and applying them similarly to suspensions. On careful consideration it becomes apparent that a suspension of cells is a different, and in a sense a more complicated, element of electrical resistance than is a block of tissue, and that the interpretation of resistance measurements requires a rather special treatment.

DISCUSSION OF METHOD

The first fact that emerges from a critical consideration of a suspension of cells is that one is measuring, in part at least, the resistance of the suspending medium as well as that of the suspended cells. It therefore becomes necessary to determine the changes that may occur in the resistance of the solution under any given experimental conditions. Further it is necessary to establish the ratio between the volume of cells and the volume of solution in the suspension, and the relation of that ratio to the resistance of the system. And finally it is important to know what significance the size and shape of cells may have in determining the total resistance.

Even when all these factors are determined there still remains the question—to what extent does the resistance of the cell

actually measure the permeability of the cell membrane? If a direct current were used, the resistance, and consequently the conductivity, of the cell could by hypothesis be considered to be a direct measure of permeability, since conductivity would be conditioned by the direct migration of ions. But in practice the use of a direct current is not feasible because of polarisation effects. To obviate these effects an alternating current is used. The theoretical advantage of an alternating current lies in the hypothesis that under the influence of such a current ions do not migrate bodily, but vibrate symmetrically, and that the current flows by the passage of charges from ion to ion down the line of fall of potential. On this assumption there seems to be no necessity and no justification for supposing that the passage of a current through a cell has any direct relation to the permeability of that cell, but depends rather on its dielectric constant. In practice, however, a true alternating current is rarely attained. The mathematical expressions for the growth and fading of a current in an ordinary induction coil show that the current fades much more rapidly than it develops. This fact is emphasised in many books on physics. Therefore the current that is obtained from a coil and interruptor does not follow a sinusoidal curve, as an alternating current should, but is, in effect, a series of unidirectional shocks. It is possible to compensate for these factors, and apparatus has been devised, and is used in modern physical laboratories which does give a true sinusoidal current, but, as far as I know, it has not as yet been applied to biological investigation. It seems not improbable, therefore, that the results that have been obtained with resistance measurements of tissues and cell suspensions, and which have been interpreted as measuring the permeability of cells to ions, have been due to the fact that these measurements have been made with what was virtually an intermittent direct current, and that the use of a true alternating current might yield results of quite a different nature.

EXPERIMENTAL

At the outset it has to be stated that the results herewith presented are provisional in character because the modern appara-

tus referred to above was not available, and measurements have been made with a simple Kohlrausch bridge.

In these experiments suspensions of *Bacillus cereus* were used. The organism was grown by means of surface inoculation on beef-peptone agar, with an incubation period of twenty-four hours at 20°C. The membranous growth was washed off with a balanced Ringer solution of the following composition:

	cc.
1/8 M NaCl	960
1/8 M CaCl₂	15
1/8 M KCl	25
	1,000

The solution was buffered with 4 drops per liter of saturated $Na_2 HPO_4$ solution, the resulting pH being 7.2.

The bacterial cells were washed by repeated centrifuging and resuspension until the resistance of the suspension attained a constant value, and the resistance of the supernatant liquid after centrifuging was the same as the resistance of the pure solution. Four centrifugings were usually sufficient. Growth from 35 agar plates was found to yield 7.5 cc. of centrifuged cells, and this volume was suspended in 7.5 cc. of solution, yielding 15 cc. of suspension. A rigid electrolytic cell (cell constant 0.3072) could be immersed in the suspension and withdrawn from it. It was thus possible to make measurements actually in the centrifuge tube, the bacteria remaining in the same vessel throughout an experiment. Temperature was regulated by means of a simple water bath, heated with an electric bulb, and was controlled by means of a sensitive thermometer inserted into the bacterial suspension. Maximum fluctuations amounted to 0.05°C.

The changes in resistance that occurred when the suspension was killed by heat are shown in figure 1. The suspension was autoclaved at 15 pounds for ten minutes. The continuous lines represent the resistance of the suspension, the dotted lines the resistance of the suspending medium after centrifuging. It is seen that the resistance of the suspension fell considerably after heating, but this was accompanied by a corresponding drop in

the resistance of the suspending medium.   On continued resuspension in 1/8 M Ringer the resistance of the suspension again rose but did not attain the value of the resistance of the living suspension.   This probably was due to the change in volume produced by heating.   Before heating, the volume of the centrifuged cells was 7.5 cc., after heating it was 5 cc.   These results can be explained if it is assumed that the death of the cells by heat resulted in a destruction of the impermeability of the cell membrane, and that there was consequently free diffusion of electro-
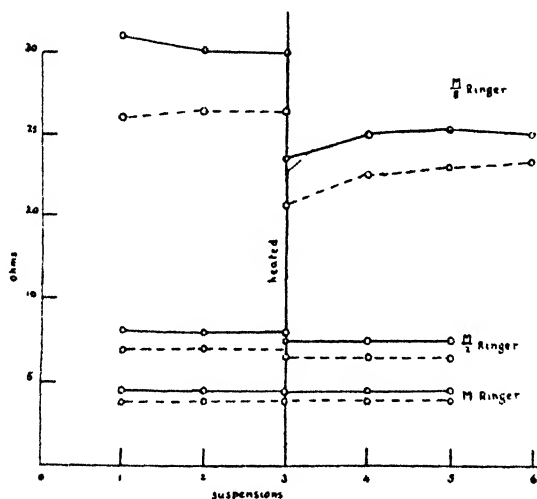
FIG. 1. EFFECT OF HEAT UPON RESISTANCE OF SUSPENSIONS OF B. CEREUS IN VARYING CONCENTRATIONS OF RINGER SOLUTIONS

Continuous lines indicate resistance of suspension; dotted lines, resistance of supernatant.

lytes in and out of the cell.   If the external solution is hypotonic to the cell contents, then there would be a diffusion of salts out of the cell, and the resistance of the suspension would drop owing to an increase in the concentration of salts in the suspending liquid.   On repeated resuspension the concentration of salts would again be lowered, and the resistance raised.   If this hypothesis is accepted then it becomes evident that in a hypertonic solution the process would be reversed, salts would diffuse into the cell,

and the resistance of the suspension and of the suspending medium would rise. A number of experiments were carried out attempting to demonstrate this phenomenon, but no rise in resistance was observed. In 1/2 M Ringer there was still a slight drop in resistance (fig. 1) in M Ringer no change in resistance was observed, and in higher concentrations up to 2 M there was also no change.

In considering these results it is important also to examine the relation of resistance of a pure solution to the concentration
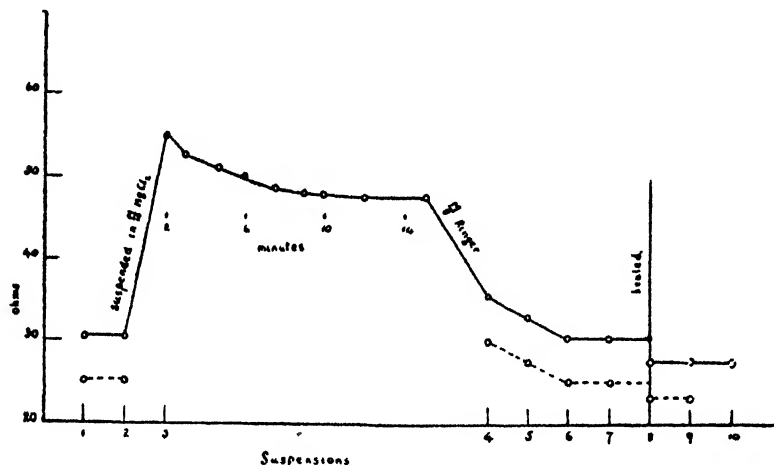
FIG. 2. EFFECT OF MERCURIC CHLORIDE AND HEAT UPON RESISTANCE OF SUSPENSIONS OF B. CEREUS

Continuous lines indicate resistance of suspension; dotted lines, resistance of supernatant.

of the salts which it contains. It is well known that resistance plotted against concentration yields an exponential curve, and that at concentrations greater than about 1/4 M resistance is very much less sensitive to changes in concentration than it is at greater dilutions. It follows therefore that in the experiments in which 1/2 M and M Ringer were used, changes in concentration may have occurred of the same order of magnitude as in the case of dilute solutions,—these changes corresponding however, to such small variations in resistance that the apparatus used was not sensitive enough to record them.

Figure 2 shows the resistance curve of a suspension which was killed with mercuric chloride, and then resuspended repeatedly in 1/8 M Ringer. The death of the cells in mercuric chloride solution was accompanied by a drop in resistance, and, incidentally, the death curve is a typical diffusion curve. When the dead cells were resuspended with washing in Ringer it was found that the resistance of the dead suspension was identical with the resistance of the living suspension, viz., 30.5. The dead suspension was then heated, and a drop in resistance resulted, but this drop was less by 4 ohms than the drop which occurred when a living suspension was killed by heat. The heating caused a reduction in cell volume from 7.5 to 4 cc., and this reduced volume is believed to account for the drop in resistance.

DISCUSSION

It is apparent from these results that changes in the resistance of the suspension are always accompanied by changes in the resistance of the suspending medium, and that the bacterial substance offers a seemingly constant resistance to an electric current, a resistance which is quite independent of the permeability or impermeability of any hypothetical enveloping membrane, and which is not affected by the death of the cell. It would seem, however, that the living cell does possess a mechanism for opposing changes in the osmotic pressure of the surrounding medium, and that this mechanism is broken down at death, thus permitting the free diffusion of salts under a concentration gradient. This phenomenon was observed both in suspensions killed by heat, and in those killed with mercuric chloride. Heating, however, causes in addition a reduction in cell volume, due possibly to the coagulation and dehydration of proteins, and this shrinkage serves to decrease still further the resistance of the suspension.

In conclusion it is desired to emphasize the distinction between the migration of ions under the influence of a potential gradient, and their migration under the influence of an osmotic gradient. The one is conductivity, the other diffusion. Diffusion, we know, is conditioned by the permeability of the cell, we do not

know what actually determines conductivity. There are no indications in any available experimental data that the resistance, and therefore the conductivity, of bacterial cells is affected or modified by external conditions or experimental manipulation. But there is ample evidence to support the conclusion that the bacterial cell is comparatively sensitive to osmotic changes in its environment, and the careful and accurate measurement of the electrical resistance of the suspension and the suspending medium offers a valuable method for investigating these phenomena.

ACKNOWLEDGMENT

After the completion of these experiments the work of Green and Larson (Jour. Infect. Dis., 1922, 30, 550) came to the writer's notice. These investigators, working with suspensions of the colon bacillus, arrived at essentially the same results and conclusions. The data presented in this paper offer an independent confirmation of their findings.

REFERENCES

Brooks, S. C. 1925 Jour. Gen. Physiol., 7, 349.
Gray, J. 1916 Phil. Trans. Roy. Soc., Ser. B, 207, 481.
Osterhout, W. J. V. 1918 Jour. Biol. Chem., 36, 485.
Osterhout, W. J. V. 1919 Jour. Gen. Physiol., 1, 409.
Osterhout, W. J. V. 1920 Jour. Gen. Physiol., 3, 145.
Shearer, C. 1919 Jour. Hygiene, 18, 337.
Stewart, G. N. 1899 Jour. Physiol., 24, 211 and 356.
Stewart, G. N. 1909 Jour. Pharmacol. and Exper. Therap., 1, 40.

A RAPID METHOD FOR THE MACROSCOPIC AGGLUTINATION TEST[1]

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While using the Kahn Precipitation Test and especially the quantitative procedure, the possibility suggested itself of applying similar principles of concentration to the demonstration of agglutinins. If an immediate agglutination reaction could be obtained by an accurate, quantitative method, the advantages would at once be apparent. Working along these lines, a rapid method for the macroscopic agglutination test has been developed.

The method involves concentration of the antigen, the use of small amounts of both antigen and serum, together with thorough mixing. Results read within five minutes after adding serum to suspension correspond to those obtained by a standard method requiring from eighteen to twenty-four hours.

EXPERIMENTAL

The first experiments were made with 0.15 cc. of varying dilutions of an antityphoid serum (1:20 to 1:1000) and 0.05 cc. of a very heavy killed suspension of *B. typhosus.* These were mixed in small test tubes (75 by 13 mm.), shaken vigorously for two minutes, 0.5 cc. saline added and read—thus following the quantities and mechanics of the Kahn Precipitation Test.

There was complete agglutination throughout, with no clumping in the control (0.15 cc. saline plus 0.05 cc. suspension). This experiment was repeated with *B. typhosus* and *B. paratyphosus*

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A and B for both homologous and cross agglutination, this time using 0.1 cc. of suspension and 0.1 cc. diluted serum. Agglutination was prompt and specific. The test was then applied to the pneumococcus with even more marked results. Agglutination by the rapid method with this organism is striking. The clumps are few and large and, with vigorous shaking, stick to the sides of the tubes leaving the liquid perfectly clear.

There was now apparent a definite need for a quantitative comparison of the rapid method with a standard macroscopic agglutination test. A test which has been in use in this laboratory for many years consists of 0.5 cc. of suspension plus 0.5 cc. of diluted serum in small pointed tubes, incubated eighteen to twenty-four hours at 37°C. The following system was therefore adopted:

For the standard method. One-half cubic centimeter suspension + 0.5 cc. diluted serum, in agglutination tubes (90 by 8 mm., tapered at the end). Mix well, incubate at 37°C. for eighteen to twenty-four hours.

For the rapid method. One-tenth cubic centimeter suspension (containing five times as many organisms per cubic centimeter) + 0.1 cc. diluted serum (five times more concentrated than for the standard method) in small test-tubes (75 by 13 mm.). Shake two minutes and add 0.8 cc. saline to make the total volume 1 cc.

Thus we have the *same amount of serum* and the *same number of organisms* in each test, for example:

In the standard method: 0.5 cc. of serum diluted 1:50 contains 0.01 cc. of undiluted serum.

In the rapid method: 0.1 cc. of serum diluted 1:10 contains 0.01 cc. of undiluted serum.

Because the majority of us are in the habit of thinking of agglutinin titers in terms of dilutions, we will call this a dilution of 1:100, meaning that there is actually present 0.01 cc. of undiluted serum, regardless of the total volume.

Also, in the standard method: 0.5 cc. of suspension containing 2 × organisms per cubic centimeter = × organisms

In the rapid method: 0.1 cc. of suspension containing 10 × organisms per cubic centimeter = × organisms

The suspension containing $2\times$ organisms must be of such density as will give maximum agglutination by the standard method with a given organism.

TABLE 1

Table of dilutions for comparative tests

	AMOUNT OF SERUM IN:		EQUIVALENT AS EXPRESSED IN DILUTIONS IN:	
	0.1 cc.	0 5 cc	0.1 cc	0.5 cc.
	cc.	cc.		
A 1:2 1.0 cc. undiluted serum + 1.0 cc. saline	0.05		1:20	
B 1:4 0.2 cc. A + 0.2 cc. saline	0.025		1:40	
C 1:8 0.1 cc. A + 0.3 cc. saline	0.0125		1:80	
D 1:10 1.0 cc. A + 4.0 cc. saline	0 01	0.05	1:100	1:20
E 1:20 0.2 cc. A + 1.8 cc. saline	0 005	0 025	1:200	1:40
F 1:40 1.0 cc. D + 3.0 cc. saline	0.0025	0.0125	1:400	1:80
G 1:50 1.0 cc. D + 4.0 cc. saline	0 002	0.01	1:500	1:100
H 1:100 1.0 cc. G + 1 0 cc. saline	0.001	0.005	1:1000	1:200
I 1:200 1 0 cc. G + 3 0 cc. saline		0 0025		1:400
J 1:250 1.0 cc. G + 4 0 cc. saline		0 002		1:500
K 1:500 1.0 cc. J + 1.0 cc. saline		0.001		1:1000

It was also thought advisable to use the same series of dilutions for both tests.   The serum dilutions were made in a separate set of test-tubes, using 1-cc. pipettes and a different pipette for each dilution.   Table 1 gives an example of such a series.

Typical results with the pneumococcus by the two methods, as outlined above, are given in table 2.   The same agglutination titer is obtained in two minutes by the rapid method as by the standard method.

It is well known that, with a potent serum and a highly agglutinable suspension, almost immediate clumping will occur in the

TABLE 2

Agglutination results by rapid and standard methods with the pneumococcus

SUSPENSIONS	METHOD	1:20	1:40	1:80	1:100	1:200	1:400	1:500	1:1000	Control
ANTIPNEUMOCOCCIC SERUM TYPE II										
Type II	R	+++	+++	+++	+++	+++	++++	++++	–	–
	S	+++	+++	+++	+++	+++	+++	+++	++	–
Type I	R	–	–	–	–					–
	S	–	–	–	–					–
Type III	R	–	–	–	–					–
	S	–	–	–	–					–
ANTIPNEUMOCOCCIC SERUM TYPE III										
Type III	R	+++	+++	+++	+++	+++	+++	++	++	–
	S	+++	+++	+++	+++	+++	+++	++	++	–
Type I	R	–	–	–	–					–
	S	–	–	–	–					–
Type II	R	+	–	–	–					–
	S	–	–	–	–					–

R = rapid method; S = standard method; $++++$ = complete agglutination with all organisms clumped and fluid clear; $+++$ = marked agglutination with nearly complete clumping but fluid not quite clear; $++$ = partial agglutination; $+$ = slight agglutination; $-$ = no clumping and no clearing.

lower dilutions by any method.   The question now arose, whether the *limit of agglutination* could be obtained by using the 0.5 cc. quantities of the regular method in larger tubes with two minutes' shaking.   To determine this the following experiments were made.

Antipneumococcic sera, Types II and III, were tested against homologous suspensions, (1) by the eighteen-hour method in agglutination tubes, each tube shaken separately, (2) same quantities in small test-tubes, shaken two minutes, and (3) rapid method, serum and antigen concentrated.

There was some agglutination by the regular method after thorough mixing in agglutination tubes and considerable after two minutes' shaking in test-tubes, but the *limit of agglutination* is not reached as it is when the reagents are concentrated as for the rapid method.

In order to determine the effect of concentration of one of the reagents as compared with concentration of both, these experiments were conducted.   Test I: To 0.1 cc. of varying dilutions of serum was added 1 cc. of suspension (diluted with saline to contain the equivalent of 0.1 cc. of heavy suspension).   Test II: To 1 cc. of varying dilutions of serum (10 times as dilute as in Test I) was added 0.1 cc. heavy suspension.   Test III: To 0.1 cc. of varying dilutions of serum was added 0.1 cc. heavy suspension.

Thus the same quantities of serum and the same number of organisms were contained in each of the three series.   All were shaken two minutes and 0.9 cc. saline added to Test III to make it equal in volume to the others.

Tables 3 and 4 give typical results of such tests with the pneumococcus and *B. typhosus*.   There is some agglutination by each method, but again the titer is not reached and the agglutination is by no means as pronounced as when both serum and antigen are concentrated.

Finally, and most important, the method of shaking has considerable effect on agglutination, not only on the titer but also on the quality of the clumping.   This is particularly noticeable with members of the typhoid group.   Rapid, vigorous shaking,

TABLE 3

Effect of concentration on agglutination with pneumococcus Type III

	1:20	1:40	1:80	1:100	1:200	Control
			RESULTS AFTER 2 MINUTES' SHAKING			
1. Serum concentrated (0.1 cc. serum + 1 cc. suspension)	++++	+++	+++	+++	+	—
2. Suspension concentrated (1 cc. serum + 0.1 cc. suspension)	+	+++	+++	++	—	—
3. Both concentrated (0.1 cc. serum + 0.1 cc. suspension)	++++	++++	++++	++++	++++	—
			ALL TESTS AFTER 18 HOURS AT 37°C.			
1.	+++	+++	+++	+++	+++	—
2.	+++	+++	+++	+++	+++	—
3.	+++	+++	+++	+++	+++	—

TABLE 4

Effect of concentration on agglutination with B. typhosus

	1:100	1:200	1:400	1:500	1:1000	1:2000	1:2500	1:5000	Control
				RESULTS AFTER 2 MINUTES' SHAKING					
1. Serum concentrated	+++	+++	+++	+++	+++	++	—	—	—
2. Suspension concentrated	+++	+++	+++	+++	++	—	—	—	—
3. Both concentrated	+	+	++++	++++	++++	++++	++++	+++	—
				ALL TESTS AFTER 18 HOURS AT 37°C.					
1.	+++	+++	+++	+++	+++	+++	+++	++	—
2.	+++	+++	+++	+++	+++	+++	+++	++	—
3.	+++	+++	+++	+++	+++	+++	+++	+++	—

such as is used in the Kahn test, produces fine, hard clumps in place of the large loose flakes to which we are accustomed, and the titer is not as high as by the standard method.   However, by using a slow but thorough mixing, characteristic clumping is produced and the results check with a standard method.   *The rack is inclined to an angle of nearly 90° and shaken slowly so that the mixture of serum and antigen flows up the tube for about 1 inch.*   Upon this method of shaking depends the accuracy of the results and the ease of reading.

Clumps once formed may be so broken up by hard shaking as to make the reading of complete agglutination considerably lower.   Table 5 illustrates this point and the effect of slow and rapid shaking.

TABLE 5
Effect of shaking on agglutination of B. typhosus

	1:100	1:200	1:400	1:500	1:1000	1:2000	1:2500	1:5000	1:10000	Control
1. Rapid shaking for two minutes	++++	++++	++++	++++	+++	+	+	+	−	−
2. Slow shaking for two minutes	++++	++++	++++	++++	++++	++++	++++	+++	−	−
3. Test 2 reshaken rapidly for two minutes after addition of saline	++++	++++	++++	++++	+++	++	+	+	−	−

From many experiments with this technic we are convinced that the same agglutination results can be obtained by the rapid method within five minutes after mixing serum and antigen as by a standard method after eighteen to twenty-four hours.

APPLICATION OF RAPID METHOD

All of the details of the method have by no means been worked out and each organism presents a problem of its own, just as has always been the case in serological tests, so that this is in the nature of a preliminary report only.   But a few of the applications of the methods may be briefly mentioned.

Experimentally, it may be used wherever agglutination tests are applied.   The rapid method lends itself particularly well to

research because of the accuracy which can be attained and because of the number of experiments which can be completed in a short time, thus facilitating repetition and variation. Clinically, the test may be used whenever agglutination is employed for the diagnosis of bacterial infection.

We have used it to determine the serum agglutinin titre of animals under treatment, as an aid in the identification and typing of strains, for the rapid checking of cultures, for absorption of agglutinins, and for the diagnosis of infectious diseases.

I. Determination of serum agglutinin titer of animals under treatment

Animals on treatment for the stimulation of agglutinins may be sample-bled, their serum tested, and results obtained immediately, thus allowing treatment to be continued without delay or bleeding to be made at the exact point desired.

In this connection comparative tests by the rapid and standard methods have been made with a number of organisms against serum from animals during immunization. Throughout all these experiments killed standardized suspensions only have been used. All glassware was clean and sterile and a separate pipette was used for each serum dilution.

B. typhosus and B. paratyphosus A and B. Comparative tests by both methods made on samples of serum, for cross and homologous agglutination, from animals injected with typhoid vaccine and typhoid-paratyphoid vaccine at first showed slightly lower titer by the rapid than by the 18-hour method and the clumps were finer, but with slow shaking, typical typhoid agglutination is obtained and the titers check very closely.

The suspensions are made from sixteen to eighteen-hour agar growths emulsified in physiological salt solution plus 0.5 per cent formalin. The density of *B. typhosus* is 1000 million organisms per cubic centimeter for the regular method, and 5000 million for the rapid.

Bact. dysenteriae, Shiga, Hiss-Y, 5 types of Flexner. The suspensions are the same as for *B. typhosus*, and there is close agree-

ment in the end results, but the reactions by the rapid method are much more easily read. There is never any doubt as to the limit.

Bt. anthracis. *Bt. anthracis* is grown on plain agar at 42.5°C. for several generations to eliminate spores. A sixteen-hour growth is then emulsified in saline with 0.5 per cent formalin to a density corresponding to that of *B. typhosus* containing 2000 million per cubic centimeter for the regular method and five times as heavy for the rapid.

Agglutination with the rapid method is a little lower, but probably gives a truer titer, as the end results after incubation are difficult to read, due to the tendency of *Bt. anthracis* to settle out. Incidentally, a suspension prepared in 1918 gave excellent results by both methods.

Pneumococcus. Comparative tests with pneumococcus, Types I, II, and III almost invariably give higher results by the rapid than by the eighteen-hour method. This may be due to the concentration of serum as a result of low titer. As stated above, the clumping of pneumococcus is most pronounced. Thirty seconds' shaking is sufficient to obtain complete agglutination and no additional saline is needed to facilitate reading.

The sediment from a young broth culture emulsified in normal saline plus 0.5 per cent formalin is used.

Meningococcus. Comparative tests on serum from animals being treated with polyvalent antigens of meningococcus and from those on the four types against monovalent type suspensions show very close agreement. The tests by the rapid method sometimes require three minutes' shaking, and those by the regular method are incubated twenty-four hours.

Tests on normal sera have never given higher results by the rapid than by the standard method.

In table 6 are given typical comparative results obtained by the rapid and standard method on serum from animals being immunized to the above mentioned organisms.

We have found this a very satisfactory and reliable method for quickly determining the serum agglutinin titer of animals under

TABLE 6

Comparative results with the rapid method and a standard method

ANIMALS BEING TREATED WITH	AGAINST SUSPENSION OF	METHOD	++++	+++	++	+	−
B. typhosus	B. typhosus	R	1:2000	1:5000			1:10000
		S	1:2000		1:5000		1:10000
B. typhosus, B. paratyphosus A and B	B. typhosus	R	1:400			1:500	1:1000
		S	1:400	1:500			1:1000
	B. paratyphosus A	R	1:100	1:200			1:400
		S	1:100	1:200		1:400	1:500
	B. paratyphosus B	R	1:200	1:400			1:500
		S	1:200			1:400	1:500
Bt. dysenteriae Shiga	Shiga	R	1:2000	1:2500			1:5000
		S	1:2000	1:2500			1:5000
Bt. anthracis	Bt. anthracis	R	1:500	1:1000	1:5000	1:10000	
		S	1:500	1:2500			1:2000
Dip. pneumoniae Type I	Dip. pneumoniae Type I	R	1:100	1:200			1:400
		S	1:100				1:200
	Dip. pneumoniae Type II	Both					1:20
	Dip. pneumoniae Type III	Both					1:20

Mic. meningitidis 4 groups	Mic. meningitidis Group I	R	1:500	1:1000		1:2000	1:2500
		S	1:500	1:1000		1:2000	1:2500
	Mic. meningitidis Group II	R	1:500		1:1000		1:2000
		S	1:500		1:1000		1:2000
	Mic. meningitidis Group III	R	1:200	1:400	1:500	1:1000	1:1000
		S	1:200	1:400	1:500	1:1000	1:2000
	Mic. meningitidis Group IV	R	1:2000	1:2000		1:2500	
		S	1:1000	1:2000		1:2500	
Mic. meningitidis Group I	Group I	R	1:2500	1:5000	1:10000	1:10000	1:12500
		S	1:5000	1:5000	1:10000	1:10000	1:12500

R = rapid method; S = standard method.

treatment.   The experiments with anthrax suggest the possibility of applying the method to others of the larger organisms, suspensions of which tend to settle out rapidly.

II. Identification and typing of strains

As an aid in the identification and typing of cultures the method is convenient and rapid.   Either live or killed organisms may be used, provided only that the emulsions are heavy.   Thirty suspensions of freshly isolated strains of *B. typhosus* and two of *B. paratyphosus* A have been run against antityphoid and antiparatyphoid sera by both methods with almost identical results.   We have used it also for typing pneumococci and meningococci and for identifying *B. dysenteriae* and *B. diphtheriae*.

The method is of value in the rapid checking of cultures already identified.   It is more accurate and less dangerous for handling live organisms than a slide method.   An agglutination test not only gives one a very good idea as to the identity, type, and purity of a strain, but also indicates the condition of agglutinability of that strain.

III. Absorption of agglutinins

Comparatively little work has been done on agglutinin-absorption, but very promising results have been obtained from the few experiments made.   Formalized suspensions only have been used.   These were centrifuged and a 50 per cent suspension of packed cells made.   To 0.3 cc. of a suspension of *B. typhosus* was added 0.3 cc. of a 1:2.5 dilution of antityphoid serum in a centrifuge tube.   This mixture was shaken slowly for five minutes and centrifugalized for five minutes.   The dilution of the absorbed serum was considered 1:5.   The unabsorbed serum was diluted 1:5 and the following dilutions of each made:

```
                                                          0.1 cc.
A 1:5........................................................ 1:50
B 1:10 —0.2 cc. A + 0.2 cc. saline........................... 1:100
C 1:50 —0.1 cc. B + 0.4 cc. saline........................... 1:500
D 1:80 —0.1 cc. B + 0.7 cc. saline........................... 1:800
E 1:100—0.2 cc. C + 0.2 cc. saline........................... 1:1000
F 1:400—0.1 cc. C + 0.7 cc. saline........................... 1:4000
G 1:500—0.1 cc. E + 0.4 cc. saline........................... 1:5000
```

To 0.1 cc. of the above dilutions of each serum 0.1 cc. of suspension was added, and both series shaken for two minutes. The serum before absorption showed complete agglutination at 1:5000, and after, incomplete at 1:50.   Serum diluted 1:5 for absorption gives about the same results.

Applying the test to the pneumococcus, 0.2 cc. of undiluted serum plus 0.2 cc. of a 50 per cent suspension of packed cells was shaken for five minutes and centrifugalized for five minutes. Serum which before absorption gave complete agglutination at 1:80 with marked clumping at 1:200, after absorption showed no agglutination at 1:20.

With serum of as low a titer as the antipneumococcic serum, the amounts may be measured with a 0.1-cc. pipette, thus elim-

TABLE 7

Absorption of agglutinins

	1:50	1:100	1.500	1:800	1:1000	1:4000	1:5000	Control
B. typhosus:								
Unabsorbed serum ...	++++	++++	++++	++++	++++	++++	++++	–
Absorbed serum......... ..	+	–	–	–	–			
	1:20	1:40	1·80	1:200	1:400			
Pneumococcus Type II:								
Unabsorbed serum.......... ..	++++	++++	++++	+++	+			–
Absorbed serum	–	–	–	–	–			

inating the process of making dilutions and considerably shortening the time.   For example, 0.1, 0.05, 0.025, 0.01, and 0.005 cc. of unabsorbed serum diluted 1:2 and the same amounts of the absorbed serum, which is already 1:2, give final results of 1:20, 1:40, 1:80, 1:200, and 1:400.

Neither the minimum absorbing dose nor the most efficient dilution of serum has been determined, but we are convinced that absorption of agglutinins may be accomplished by thorough mixing of small concentrated amounts of serum and antigen within five minutes, and the time of the whole process shortened from twenty-four to forty-eight hours to less than two hours.

IV. Application to diagnosis

Clinically, the rapid method has been used for the diagnosis of the typhoid and paratyphoid fevers and the dysenteries, and for the typing of pneumococci and meningococci. The typing of pneumococci will serve to illustrate the application of the test to clinical diagnosis.

Four amounts of each type serum—0.1, 0.05, 0.025, and 0.01 cc.—are measured into three sets of four tubes each. The amounts are pipetted with a 0.1 or 0.2-cc. pipette to the bottom of the tubes. To each tube is added 0.1 cc. of a heavy suspension of the organism, which may be peritoneal washings from mouse, pure culture, or heavily infected spinal or pleural fluid. A control containing 0.1 cc. suspension and 0.1 cc. saline is included. All are shaken slowly for two minutes, 0.5 cc. saline added to each tube, and the results read.

The rapid method has never failed either with patients' serum or identifying strains where the standard method was successful and, on account of the rapidity of the reaction, freshly isolated cultures which show some spontaneous clumping may be used.

SUMMARY

A rapid method for macroscopic agglutination is described in which the reactions are sharp and well defined and check with those given by a standard eighteen-hour method.

Either killed standardized suspensions or live cultures may be used and, on account of the rapidity of the reaction, suspensions of organisms which tend to settle out on standing give good results.

In animal immunization work the serum titer can be determined at the exact point desired.

The rapidity of the reaction is of practical value in making possible the repetition of tests and variation of conditions for both diagnostic and research purposes.

REFERENCES

KAHN, R. L. 1924 The Kahn Precipitation Test for syphilis. Demonstration. Amer. Jour. Public Health, 14, 498.

NOBLE, ARLYLE 1919 A method for the production of a homogenous suspension of B. anthracis to be used in agglutination reactions. Jour. Immunol., 4, 105.

FURTHER STUDIES ON BACTERIAL ALLERGY

THE ANTIGEN INVOLVED IN PNEUMOCOCCUS ALLERGY

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I

In our studies upon the tuberculin reaction we have attempted to proceed from the premise that the phenomenon of bacterial allergy is probably a universal accompaniment of infection, and plays a perhaps important rôle in the symptomatology and tissue injury associated with bacterial toxemias. Such a conception was indicated not only by the fact that occurrences analogous to the tuberculin effects were apparent in the abortin, Mallein and typhoidin reactions, but was also suggested by previous studies by others and by ourselves with typhoid bacilli, streptococci, staphylococci and other organisms. In an investigation of streptococcus allergy which the present writers published a little over a year ago (1925), and in similar studies carried out by Dochez and Sherman (1925), it was made quite clear that the sensitization of guinea pigs can be accomplished by the injection of streptococci, and that subsequent skin sensitiveness can be elicited with all the streptococcus materials but especially with the Dick filtrate which, in manner of production and in the important attribute of heat stability, corresponds to some extent with tuberculin. We suggested at this time the possibility that such streptococcus allergy might have some relationship to certain forms of arthritis in which the association of sterile joint conditions with streptococcus infection has been a clinically recognized fact for so long.

The difficulty in experimentation upon the problem of bac-

terial allergy with bacteria other than the bacillus of tuberculosis and the abortus bacillus has been the apparent necessity of producing prolonged infections and tissue reactions in the animals, since, as it is well known, tuberculin reactions do not appear with any constancy or severity unless there is a tissue reaction in the form of a tubercle, whether this is produced by living bacilli or, as shown by Petroff with one of the writers, Zinsser and Petroff (1924) by the dead organisms. Owing to this, it has been generally assumed that true allergy represented an "infection" rather than an "injection" reaction, for reasons never entirely clear. And, indeed, it is true that however one sensitizes, either with living or dead bacteria, which are subject to relatively rapid removal by phagocytes, or with chemically extracted bacterial materials, the type of allergic reaction one obtains, though definite, is not characterized by the violence, central necrosis and frequent hemorrhage which is so often the case with the true tuberculin reaction in guinea pigs.

The experiments which are cited in this paper seem to us to explain to some extent why this has been the case. They appear to indicate, moreover, that when one injects living bacteria, dead bacteria or bacterial extracts, the antigen is in a form which does not arouse the specific response necessary for the development of complete allergy, and probably because of the speed of removal, the changes necessary for the production of this antigen cannot take place. Our basis for this statement will become apparent below.

The experiments recorded in this paper are based upon an observation made in the course of our previous studies on the tuberculin reaction in which, suspecting the probable importance of the action of tissue cells upon the bacterial materials in the tubercle, we investigated the action upon the skin of guinea pigs of various intradermally injected bacterial autolysates. In the case of the pneumococcus, which autolyzes with great ease, we observed occasional severe skin effects upon normal guinea pigs which in extent, severity, hemorrhage and occasional necrosis were quite equal to and often more severe than the most extreme tuberculin reactions. The toxic factor in such material is a heat

stable substance[1] which we believe to be identical with that with which Julianelle and Reimann (1926) obtained purpuric reactions in mice.

We at first regarded these reactions as due to the primary toxicity of the pneumococcus substances, and were at a loss to explain the irregularity with which the material produced reactions in different animals.

In the course of several months' experimentation it became apparent that when reactions did occur they were observed only in well-grown guinea pigs weighing 400 grams or more, whereas young pigs were almost invariably negative on first test.

The following table will illustrate these relations:

	NUMBER OF PIGS POSITIVE ON FIRST TEST	PERCENTAGE OF PIGS POSITIVE ON FIRST TEST	NUMBER OF PIGS NEGATIVE ON FIRST TEST	PERCENTAGE OF PIGS NEGATIVE ON FIRST TEST
		per cent		per cent
Old guinea pigs.. .. .	18	54	15	45
Young guinea pigs	0	0	48	100
Total... . ..	18	22	63	77

Two alternative explanations for this observation are possible. Either the autolysate has a primary toxicity which is neutralized or destroyed in some manner in the younger guinea pigs—perhaps destroyed by a more potent enzyme activity—or else the reaction is not one of primary toxicity but depends upon some form of sensitization which has spontaneously occurred in a percentage of the older animals.

The former explanation, which seemed to us at that time the more likely, was investigated in the following manner:

Young guinea pigs likely to be negative and old guinea pigs that we knew to be strongly positive were bled and, since it would have obviously vitiated the experiment to test them just before bleeding, skin tests were done upon them immediately after bleeding, so that their negative or positive nature at the time of bleeding would be apparent on the following day. The serum was obtained and

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[1] The active substance is not destroyed by boiling at pH 7, and is weakened but not wholly inactivated by autoclaving at 10 pounds pressure for fifteen minutes.

mixtures then made, two parts of autolysate being added to one part of serum. The mixtures were set into the incubator for from one to three hours and skin reactions carried out upon a series of known positive guinea pigs. Controls were done on these pigs with autolysate similarly diluted with salt solution and kept in the incubator for the same time.

We did a great many experiments according to this scheme, varying somewhat the relative quantities, and have since made further tests of a like nature for the purpose of determining degrees of neutralization. In all of them we have obtained consistent results to the effect that the serum of negative animals has absolutely no neutralizing effect upon the toxicity of the autolysate for sensitive animals. It was perfectly obvious, therefore, that the negative results obtained in young guinea pigs were not due either to a neutralization or to a proteolytic destruction of the autolysate by the serum constituents of the animals.

The remaining alternative, namely, the possibility that the reactions observed in the guinea pigs represented a form of sensitization or allergy, was investigated by observing some thirty animals of various ages repeatedly injected in the course of several months and by analyzing fluctuations of reaction. As a result of this study it became quite apparent that the severe skin reactions we had obtained with the pneumococcus autolysates were manifestations of hypersensitiveness which could be elicited at will by a variety of methods.

The following tables illustrate the relations:

Slow sensitization by intradermal injections

NUMBER OF PIG	OCTOBER 18	OCTOBER 19	OCTOBER 20	OCTOBER 21	OCTOBER 22	OCTOBER 23	NOVEMBER 12
1	0 2 cc.	Practically negative, 0.2 cc.	Practically negative, 0.2 cc.	Slight redness, 0.2 cc.	Moderate, 0.2 cc.	Moderate	Strong
2	0.2 cc.	Practically negative, 0.2 cc.	Practically negative, 0.2 cc.	Slight redness, 0.2 cc.	Practically negative, 0.2 cc.	Moderate	Severe

Slow sensitization by intraperitoneal injections of autolysate

NUM-BER OF PIG	OCTOBER 18	OCTOBER 19	OCTOBER 20	OCTOBER 21	OCTOBER 22	NOVEMBER 12
3	0.2 cc.	0.2 cc., negative	0.2 cc., negative	0.2 cc., negative	0.2 cc., negative	Severe
4	0.2 cc.	0.2 cc., negative	0.2 cc., negative	0.2 cc., negative	0.2 cc., negative	Strong

The above extract from our records is typical of the slow sensitization which is occasionally obtained when animals receive repeated negative intradermal tests on successive days. In many cases moderate reactions may begin after the 5th and 6th injections and develop into powerful reactions three or more weeks after the last injection.

Sensitization after one intradermal injection of 0.2 cc.

NUMBER OF PIG	SEPTEMBER 8	SEPTEMBER 16	SEPTEMBER 24	SEPTEMBER 29	OCTOBER 26
5	Negative	Extreme		Severe	Very severe
6	Negative		Strong		
7	Negative		Strong		

The above protocol shows the speed and severity with which sensitization may develop in normally negative animals after a single intradermal injection of 0.1 to 0.2 cc. of the autolysate.

Rapid sensitization intradermal. Desensitization after one large dose intraperitoneal

PIG	OCTOBER 28	OCTOBER 29	OCTOBER 30	OCTOBER 31	NOVEMBER 1	NOVEMBER 2	NOVEMBER 3	NOVEMBER 4	NOVEMBER 5
8	0.1 cc.	Practically negative, 0.1 cc.	Very slight, 0.1 cc.	Much greater, 0.1 cc.	Mild, 0.1 cc.	Moderate 0.1 cc.	Moderately strong	0.1 cc. I.D. 1.0 cc. I.P.	Negative
9	0.1 cc.	Practically negative, 0.1 cc.	Practically negative, 0.1 cc.	Practically negative, 0.1 cc.	Mild, 0.1 cc.	Severe, 0.1 cc.	Severe	0.1 cc. I.D., 0.1 cc. I.P.	Practically negative

The foregoing table represents two animals of a not inconsiderable series which indicated the speed with which sensitization may occur after daily intradermal administrations of the autolysate, a speed quite out of keeping with the ordinary laws of anaphylaxis; and this seems to us of considerable importance in connection with our view that allergic sensitization may occur in the course of an infectious disease.

This table also illustrates the sudden desensitization which may occur when considerable amounts of the autolysate are injected into sensitive animals intraperitoneally. It is a peculiarity which we have not yet been able to explain that, in spite of the extraordinary severity of the reactions upon the skin, a sensitive animal may be given five or even ten times the skin dose intraperitoneally without injury, a matter which, in view of later experiments, may be partially explained by a slight neutralization of the antigen by the peritoneal exudate, by possible dilution and perhaps by the distribution of the antigen by peristaltic action over a large serous surface.

A sensitive animal injected with 1 cc. of the autolysate was autopsied by us several hours after the injection without finding more than a moderate injection of the peritoneum and the serous coverings of the gut.

The following table still further illustrates the degree to which our reactions correspond with phenomena of hypersensitiveness in general:

Slowly developing sensitiveness followed by desensitization and return to sensitive condition

PIG	JUNE 18–30	JULY 7	JULY 8	JULY 9	JULY 14	JULY 15	SEPTEMBER 9
10	6 negative tests	Moderate	Strong	Negative	Negative	Negative	Very strong

PIG	SEPTEMBER 10	SEPTEMBER 22-28	OCTOBER 11	OCTOBER 12	OCTOBER 18	OCTOBER 19	OCTOBER 20	OCTOBER 21	NOVEMBER 22
11	Negative	6 daily injections 0 2 cc 1 D with severe but gradually decreasing reactions	0 2 cc , intradermal	Strong reaction	0 2 cc., intradermal	Strong reaction, 2 0 cc intraperitoneally	0 2cc , intradermally	Negative	Severe reaction

These two guinea pigs, chosen from our records, illustrate the occasional desensitization which occurs in guinea pigs that have several times strongly reacted in rapid succession when a further reaction is done upon them on the day following the last strong reaction. It also illustrates that such animals, given a rest of three weeks to a month, again become strongly reactive.

The examples of our experiments cited in the above series show that reactions in guinea pigs to the pneumococcus autolysate are examples of true allergic sensitization, and that they follow closely the laws of sensitization in regard to relation to previous injections, desensitization, and the return of desensitized animals to the hypersusceptible state. They differ from the usual observations made with hypersensitiveness in the fact that sensitiveness may be developed with unusual rapidity.

II

It appears, then, that we have observed a violent allergic skin reaction in animals sensitized to a pneumococcus autolysate which differs from the most extreme tuberculin reactions largely in being more severe, since it is surrounded by a much more extensive area of oedema and may show central ulcerations that do not heal for several weeks. It is puzzling, however, that neither we nor others have obtained reactions of this type in the many previous experiments in which whole bacteria have been

used throughout. It is true that sensitization to pneumococci has followed injections of whole bacteria and bacterial nucleo-proteins, and that similar results have been obtained with streptococci, typhoid bacilli and staphylococci; but never have the reactions shown anything like the extreme severity, hemorrhage and necrosis that we have obtained with autolysates.

FIG. 1. GUINEA PIG TESTED ON SEPTEMBER 10, WITH NEGATIVE REACTION

A. Reaction from intradermal injection of 0.2 cc. autolysate, September 22.
B. Reaction from intradermal injection of 0.2 cc. autolysate, September 23.

It would appear that in the course of what we speak of loosely as "autolysis" there may be liberated an antigen more truly representing the substances liberated in the body in the course of infection than do any *in vitro* products, and that in attempts to extract bacteria with alkali, antiformin, etc., a certain amount of change or denaturization takes place by which the antigen is destroyed or modified.

In approaching this question it may be well to say a few words about the production of the autolysates as we have used them.

Pneumococci were grown on pie plates of blood agar, the cultures washed off with salt solution, once washed in the centrifuge and the sediment emulsified in salt solution at reactions ranging from 6.8 to 7.2. Thymol, toluin, or 0.5 per cent carbolic acid were added to various specimens, and the total sediment from 5 or 6 pie plates taken up in rarely more than 6 cc. total volume. Such thick suspensions began to clear up considerably within twenty-four hours, more so after three days in the incubator. They never became entirely clear, but at the end of this time showed no Gram positive cocci. The active sustance in several tests was found in the supernatant fluid.

It will be seen from the manner of production that among other things these autolysates represented a concentration of pneumococcus substance far stronger than anything obtained from the ordinary bacterial cultures or bacterial suspensions used in the previous forms of experimentation, and the quantitative factor must, therefore, be taken into account in appraising the results.

The question immediately arises whether we were dealing with a true autolytic product or with a simple bile solution of the organisms, and comparative tests were made. Experiments were carried out to determine whether the reactions in sensitive guinea pigs are in response to the unaltered dissolved pneumococcus substances, or whether a certain amount of autolysis is necessary to produce reactions. An example of such experiments is as follows:

Four guinea pigs likely to be sensitive were selected. A fresh lot of pneumococcus was washed off sixteen pie plates with salt solution and immediately centrifuged. Sediment taken up in 5 cc. of 0.5 per cent carbolyzed salt solution was divided into two lots. To one of these was added one-tenth volume of 1:10 taurocholate. To the other was added an equivalent volume of 0.5 per cent carbolic salt solution. Both were placed in the water bath and left there with two separate additions of 1 cc., respectively, of taurocholate solution to one and one-half carbolic salt solution to the other. After two hours in the water bath and about an hour in the ice-box, tests were done on the four guinea pigs with 0.2 cc. of each of the above, and another control injection done on each pig with a similar amount of a seven-day old thymol autolysate. In all these pigs the bile solution gave fair reactions, but in all but one that material to which no bile had been added, and which was quite turbid when the bile solution was clear, gave

stronger reactions than the bile solutions. In this one animal the two reactions were approximately equal. The old autolysate was the weakest of all.

From the above protocol it is clear that bile dissolved pneumococci give reactions in every way comparable to those produced by the pneumococcus autolysate.

The following is another table in which a similar comparison was made.

Comparison of speed of formation of active antigen by autolysis and by bile solution

Equivalent suspensions of pneumococci:

    A. Twenty-four hour autolysate, heated at 70° for ten minutes.
    B. Pneumococcus suspension heated after fifteen minutes at 70° for ten minutes.
    C. Bile solution heated after twenty-four hours at 70° for ten minutes.
    D. Bile solution heated after fifteen minutes at 70° for ten minutes.

Reactions on four sensitized pigs:

    (1). A. Mild reaction
        B. Negative reaction
        C. Severe reaction
        D. Severe reaction

    (2) A. Mild reaction
        B. Negative reaction
        C. Strong reaction
        D. Strong reaction

    (3) A. Mild reaction
        B. Negative reaction
        C. Moderate reaction
        D. Moderate reaction

    (4) A. Slight reaction
        B. Negative reaction
        C. Slight reaction
        D. Strong reaction

It is apparent from this that the bile solutions are often more active upon sensitive guinea pigs than are autolysates produced in the usual way. Moreover, if the pneumococci are heated to 70°C. within fifteen minutes after suspension in salt solution, enzyme action is stopped and the autolysate does not form. In the case of the bile preparations—on the other hand—heating within the same period does not prevent the liberation of the

responsible antigen—a process which has apparently been considerably accelerated by the bile.

Since it would unnecessarily lengthen our paper, we abstain from citing a considerable number of experiments from which it is apparent that it is possible to sensitize with similarly prepared bile solutions, as well as with the autolysates.

The bile solution, then, is antigenically equivalent to the autolysate, and it remains to determine whether or not the solution by bile represents, as suggested by Atkin (1926), a speeding up of autolysis, or whether, by destroying the morphological entity of the organisms, it merely liberates an antigen which is similarly liberated by the autolytic process without the bile, but in a slower manner. We thought that we could determine this by dissolving pneumococci by other methods, grinding, freezing and thawing, hypertonic salt solution and antiformin; but in none of these experiments did we produce a material that was immunologically equivalent to either of the other two; and with the antiformin we found that an active autolysate was destroyed by solvent amounts of antiformin, this method, therefore, being useless as an approach to the problem.

We are inclined to favour the view of Atkin in assuming that bile solution signifies a speeding up of autolysis for the following reasons: in the first place, heating pneumococci to 65° or over inhibits both the action of bile and at the same time stops autolysis; heating suspensions of pneumococci for ten minutes at temperatures ranging between 50° and 70°C. shows that both autolysis and bile solution are interfered with to an increasing extent as the temperature is raised, and that interference with the two effects is parallel. Furthermore, heated suspensions can be partially dissolved by adding to them either autolyzed pneumococci or bile-dissolved suspensions, indicating that the heat neither destroys the antigen nor renders the pneumococcus insoluble, but merely destroys the enzyme present. We at first believed that the speed with which bile solution takes place was inconsistent with the view that it represents a hastened enzyme action, but experiments on another problem in which it has been necessary for us to test filtrates for the presence of trypsin

demonstrated a comparable speed for trypsin action under suitable conditions. We must admit, however that none of these things definitely determines whether the antigen which renders our autolysates effective is a liberated, pre-formed pneumococcus constituent, or whether it is a product of autolysis.

In taking into consideration the quantitative factor involved in our experiments, namely, that in the production of the autolysates amounts of pneumococci per volume were used which were inordinately greater than in former methods, we carried out a few experiments in which sensitization was attempted by fresh pneumococcus suspensions of equal density with those used for autolysate production, but heated immediately after suspension. The injection of such substances into guinea pigs sensitized as did the autolysate, but the reactions could not be produced in these animals with heated suspensions, an autolysate being necessary to produce the characteristic lesions. This we took to reenforce our opinion that the substance produced in the test tube by autolytic processes may be produced in the animal body by the action of the animal tissues or fluids and their enzymes, thus liberating the antigen which, in our basic experiments, was produced *in vitro*.

As to specificity, our experiments have shown that sensitization with autolysates of Type I sensitizes to autolysates of Type II, and *vice versa*, thus indicating again in another way that this variety of sensitization has no relationship to the type specific antibodies, but follows rather closely the laws of specificity recognized by Avery and Heidelberger (1925) and by ourselves as depending upon the group proteins and not upon the type antigens.

We have above alluded to our preliminary experiments upon the possible neutralization of the autolytic antigen by the sera and peritoneal exudates of sensitive animals. We have repeated these experiments on a larger scale and have found invariably that a certain definite but moderate amount of neutralization can be accomplished, but that this never amounts to complete neutralization and cannot be carried to any significant degree of serum dilution.

Attempting similar neutralizations with the sera of horses highly immunized with autolysates, our experience has been the same—slightly more neutralization with such serum than with normal horse serum—but never extraordinary and always devoid of any relationship to contents of agglutinating or other measurable antibodies.   A corollary to these observations is to be found in our experiments on passive sensitization, which were invariably negative.   We were not able to bring about passive sensitization either with the sera of sensitive animals or with the sera of highly immunized horses, whether these were rich in antibodies or not.

Similarly, sensitization was not transmitted to the young of sensitized mothers.

DISCUSSION

Our experiments seem to us to involve a number of important suggestions which may bear upon an understanding of bacterial antigens and bacterial allergy.   It appears that the type of bacterial allergy that can be obtained by the injection of ordinary amounts of dead bacteria or chemically obtained bacterial extracts is of a variety different from reactions like the typically severe tuberculin reaction in guinea pigs.

When, on the other hand, autolysates of thick suspensions of the bacteria are injected by any parenteral route, guinea pigs become skin sensitive in a manner that is often more severe than that observed in the most extreme tuberculin reaction.   There is not only an oedematous swelling of considerable size, but there is often central hemorrhage and not infrequently necrosis that leads to ulceration.   It is demonstrated, therefore, that bacterial sensitization may take a form that implies a very severe response which seems to be either specific for certain parts of the body—as the skin—or else requires a temporary concentration of the antigen, such as that which can be easily obtained only by the intracutaneous injection.

Similar sensitization can be accomplished by comparable amounts of the dead unautolyzed bacteria, but the actual skin reaction can be obtained only with the autolytic products themselves, a fact which indicates to us that the responsible antigen

is either formed or liberated during this process of autolysis. That it is formed under the influence of bile solution, if a sufficient concentration of bacteria is employed, has been demonstrated, but we hesitate to decide from this whether the antigen is preformed in the animal body, or whether—on the other hand—bile solution is a process which merely involves a facilitated autolysis.

Suitably performed, such sensitization may be brought about with extraordinary speed, originally negative animals becoming moderately positive within five, six or seven days under the influence of daily injections of 0.1 to 0.2 cc. of the autolysates.

The responsible material is heat stable and is present in the supernatant fluid if autolysates are centrifuged clear. It is destroyed by treatment with antiformin.

The reaction resembles the tuberculin reaction, not only in severity and general character, but in the fact of the heat stability of the antigen, the difficulty of passive sensitization, and the difficulty of neutralization by the sera of sensitive or immunized animals.

We are encouraged to believe that our experiments suggest an explanation of bacterial allergic phenomena in general. Autolysis and bile solution, both of them processes in which no violent or artificial chemical injuries are involved, liberate from the bacteria antigenic substances which sensitize and elicit reactions quite different from those which can be elicited by the whole cells or the chemical extracts of the same organisms. It would appear reasonable to suppose that substances analogous to our pneumococcus autolysates may be liberated from the bacteria within an infected body in the case of many organisms which are not subject to test tube autolysis, at least with the same ease with which this can be accomplished with pneumococci. Such an assumption would readily explain the well-known differences between the so-called "infection" and "injection" responses, and suggest a clue to the true nature of the tuberculin reaction, which we are now following further.

In the specific instance with which our paper deals, it of course suggests itself that reactions similar to those described in guinea

pigs may have some bearing upon the occurrences in human pneumonias, where a rapid autolysis in the lung supplies these antigens in considerable quantities, since we have shown that sensitization of guinea pigs by the daily administration of small amounts of autolysate may take place within five, six or seven days.   Indeed, the work of Weil (1916) and unpublished observations of Bigelow, (1921) both of whom studied substances which in principle must have been identical with our autolysates, indicate that sensitization to these materials takes place in the course of pneumococcus infection.

REFERENCES

ATKIN, E. E. 1926 Brit. Jour. Exp. Path., 7, 167.

AVERY, O. T., AND HEIDELBERGER, M. 1925 Jour. Exp. Med., 42, 367.

BIGELOW, G. H. 1921 Thesis submitted for the degree of Doctor of Public Health, Harvard University, May 1, 1921.

DOCHEZ, A. R., AND SHERMAN, L. 1924–25 Proc. Soc. Exp. Biol. and Med., 22, 282.

JULIANELLE, L. A., AND REIMANN, H. A. 1926 Jour. Exp. Med., 43, 87.

WEIL, R. 1916 Jour. Exp. Med., 23, 11.

ZINSSER, H., AND GRINNELL, F. B. 1925 Jour. Immun., 10, 725.

ZINSSER, H., AND PETROFF, S. A. 1924 Jour. Immun., 9, 85.

OBSERVATIONS ON THE GROWTH OF YEASTS IN PURE NUTRIENT SOLUTIONS

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Yeasts have been propagated in synthetic nutrient solutions since 1860 when Pasteur was carrying out his classic experiments. In 1901 Wildiers gave the whole field of investigation new interest by suggesting that a hypothetical substance, to which he gave the name "bios," was necessary for their fullest development. He believed that yeast would grow in a synthetic medium without this "bios," but that the development was much better when it was present. Wildiers employed a medium composed of water, sucrose, magnesium sulphate, potassium chloride, and calcium carbonate. This medium was prepared from pure salts and inoculated with *Saccharomyces cerevisiae;* multiplication was measured by loss in weight in the flasks due to CO_2 liberated. He found that small seedings produced no fermentation while large seedings did produce fermentation. He concluded that growth was stimulated, not because of an increase in the number of cells used, but by some growth promoting substance which was added when large seedings were employed. Growth was activated by the addition of a filtrate from boiled yeasts, which contained this constituent essential for yeast growth, namely, "bios." This "bios" was believed to be a substance other than sugar and mineral nutrients and absolutely essential for the growth and activity of yeasts.

It would be almost impossible to summarize various data and opinions that have been published since 1901. In a very complete review of the question, Tanner (1925), pointed out that at present there seemed to be several groups of investigators; one group denies the existence or need of a substance like "bios"

for yeasts; another group believes that "bios" is necessary, some of this group having reported the isolation of this substance; and a third group believes that yeast will grow in pure nutrient solutions without "bios" but that the addition of a "bios" containing substance may cause increased growth.

Robertson (1924) reported that continuous growth was not obtained when Robertson and Davis' (1923) synthetic medium was employed for the growth of yeasts. In order to confirm this statement, forty-six different species of yeasts were inoculated into tubes containing 10 cc. of Robertson and Davis' medium, and incubated at 30°C. for seven days. Transfers were then made (with a loop) into fresh media, and the cultures incubated for another seven days. The experiment was carried for twenty-eight weeks. The following yeasts were employed:

1. Burgundy wine
2. Champagne yeast
3. *Cryptococcus agregatus*
4. *Cryptococcus glabratus*
5. *Cryptococcus Ludwigii*
7. *Endomyces albicans*
8. *Endomyces javanensis*
9. *Monilia tropicalis*
10. *Mycoderma rugosa*
11. *Mycoderma vini*
13. Yeast from oysters—Hunter
15. *Parasaccharomyces Ashfordii*
17. *Pichia farinosus*
18. *Pichia membranaefaciens*
19. Red yeast (Snow) I—21
20. *Saccharomyces albus*
21. *Saccharomyces anomolus*
22. *Saccharomyces capsulares*
23. *Saccharomyces carlsbergensis*
24. *Saccharomyces cerevisiae*
25. *Saccharomyces ellipsoideus*
26. *Saccharomyces hominis*
27. *Saccharomyces intermedius*
28. *Saccharomyces logos*
29. *Saccharomyces mandshuricus*
30. *Saccharomyces marxianus*
31. *Saccharomyces neoformans*
32. *Saccharomyces Pastorianus*
33. *Saccharomyces spec* (Plimmer)
34. *Torula colliculosae*
35. *Torula communis*
36. *Torula cremoris*
37. *Torula datilla*
38. *Torula glutinis*
39. *Torula humicola*
40. *Torula mucilaginosa*
41. *Torula rubra*
42. *Torula sphaerica*
43. *Willia anomala*
44. *Willia belgica*
45. *Willia saturnus*
46. *Zygosaccharomyces biporus*
47. *Zygosaccharomyces chevalier*
48. *Zygosaccharomyces mandshuricus*
49. *Zygosaccharomyces pastori*
50. *Zygosaccharomyces priorianus*

After twenty-eight weeks continuous multiplication in Robertson-Davis' medium, it was found that (see table 1) out of the 46 cultures employed, 22 displayed abundant growth, 2 moderate growth and 2 scant growth. The 20 remaining cultures died

in from two to sixteen weeks. *Saccharomyces cerevisiae* (no. 24) displayed scant growth and *Saccharomyces ellipsoideus* (no. 25) abundant growth. It is interesting to note that *Saccharomyces cerevisiae*, a strain used by many workers in studies of this question, and especially by Robertson and Davis, did not find proper food substances for continued multiplication. This would suggest that one should not generalize from experiments with one species. To seek information on this question it was noted that Mitra (1917) found sodium chloride to inhibit yeast growth when present in a concentration of 0.2 mol. per liter or more, while in a concentration of 0.001 mol. per liter it stimulated yeast

TABLE 1

Observed growth at end of 28 seven-day transfers

MEDIUM	GROWTH			
	Abundant	Moderate	Scant	Apparently none
R and D with NaCl	1, 5, 9, 11, 13, 17, 18, 19, 21, 25, 26, 27, 32, 34, 36, 40, 42, 43, 44, 45, 47, 50	7, 30	24, 39	2, 4, 8, 9, 10, 15, 20, 22, 23, 28, 29, 31, 33, 35, 37, 38, 41, 46, 48, 49
R and D without NaCl	5, 11, 13, 17, 18, 19, 21, 27, 34, 36, 43, 44, 45, 47, 48, 50	7, 9, 10, 15, 24, 25, 30, 32		1, 2, 3, 4, 8, 20, 22, 23, 26, 28, 29, 31, 33, 35, 37, 38, 39, 40, 41, 42, 46, 49

growth. To partially check this an experiment was carried on, parallel to the above experiment, in which the medium used was the Robertson and Davis' medium without the sodium chloride. Sixteen out of the 46 cultures employed, showed abundant growth at the end of 28 seven-day transfers, 8 displayed moderate growth while the remaining 22 died in from two to seventeen weeks. *Saccharomyces cerevisiae* and *ellipsoideus* under the conditions of this experiment both showed moderate growth. Comparing this to the first experiment it appears that sodium chloride in the concentration used was harmful to *Saccharomyces cerevisiae* but harmless if not somewhat beneficial to *Saccharomyces ellipsoideus*.

Pichia farinosus was able to multiply equally well in either medium, which was in accordance with the microscopic observations. This one yeast was selected to represent a group that apparently grew equally well in either medium. The members of this group were nos. 5, 7, 11, 13, 17, 18, 19, 21, 27, 30, 34, 36, 43, 44, 45, 47, and 50; nos. 7 and 30 of this group were moderate growers, while the remainder were abundant growers.

Nos. 1, 26, 40 and 42 apparently needed NaCl very urgently since they did not grow in Robertson and Davis' medium without NaCl but displayed a rich growth in Robertson and Davis' medium with NaCl.

In many of the tubes (nos. 11, 13, 17, 18, 21, 43, 45 and 47) displaying rich growth, the yeasts climbed the walls of the tubes; as much as 1 cm. in some cases. This happened with yeasts cultivated with and without sodium chloride.

After the experiment was discontinued the twenty-eighth transfer (last) of the cultures was placed in the ice box. Four months later these cultures were removed and inoculated (0.2 cc. into 10 cc. medium) into the same media in which they had been previously cultured. After four days incubation at 30°C. all the cultures displayed growth which was only slightly below that obtained at the end of twenty-eight weeks.

STUDIES IN THE SALT REQUIREMENTS OF YEASTS

The object of this part of the work was to construct an inorganic salt and sugar medium which would support the continuous growth and multiplication of yeasts. After reviewing the literature on the effect of various compounds on yeasts, it was decided necessary to incorporate the following elements in the medium: C, N, O, P, K, Ca, Mg, S, Cl. Sucrose, being relatively cheap and available in a pure state, was chosen as the source of carbon. The other elements were added in the form of the following salts: NH_4Cl, $K_2HPO_4 \cdot CaCl_2 \cdot$ and $MgSO_4$. The plan of this experiment was to add first one salt then another, in various concentrations, to a 10 per cent sucrose solution and determine their power to support yeast growth and also to

determine, at the same time the most suitable concentration of the salt in question. In this manner the optimum concentration of $(NH_4)_2HPO_4$ (since phosphorus was present in the buffer, this salt was discarded) with sucrose, NH_4Cl with sucrose, and K_2HPO_4 with sucrose, were determined.

After considering a number of methods, the microscope cell method was chosen as the procedure to be used in determining the counts.

Growth in NH_4Cl and sucrose. The media consisted of sucrose, 100 grams per liter, and NH_4Cl in the following amounts per liter: 0.6, 1.2, 1.5, 1.9, 3.0, 4.0 and 6.0 grams. This set of media was made in liter quantities, autoclaved at 15 pounds for twenty minutes, and placed in sterile 100 cc. Erlenmeyer flasks in 50 cc. quantities. The flasks were inoculated with 0.05 cc. of a water suspension of a twenty-four-hour culture of yeast. The yeasts were *Pichia farinosus, Saccharomyces albus, Saccharomyces cerevisiae, Saccharomyces ellipsoidus, Saccharomyces marxianus,* and *Saccharomyces Pastorianus.* Immediately following the inoculation the number of cells per cubic centimeter in the various suspensions was determined by a Haemacytometer (Zappert-Neubauer Ruling). From this count the initial count of the flasks was determined. The count was made by counting 210 small squares (35 squares to a field and 6 fields). A magnification of 500 was employed. Haemacytometer counts were made on the flasks every day for seven days then transfers were made. In making the transfers 0.1 cc. was transferred to 50 cc. of fresh medium. These seven-day transfers were made and counts determined until cultures gave evidence of dying out or evidence that they would apparently display continuous growth. In this manner the most suitable concentration of NH_4Cl with sucrose was found to be 0.12 per cent by weight or 1.2 grams per liter. Fulmer (1925) found the optimum concentration of NH_4Cl for yeast growth at 30°C. was 1.88 gram per liter (0.0353N). Differences in the methods of approaching the problem, the yeasts used, and the method of determining the growth or multiplication might account for some of the difference between the above figure and the one obtained in this particular investigation.

Growth in K_2HPO_4 and sucrose. The same procedure was employed in determining the optimum concentration of K_2HPO_4 for yeast growth and multiplication as was used in the work on NH_4Cl. Taking the yeasts in general 0.05 per cent K_2HPO_4 appeared to bring about the most vigorous growth.

Construction of a new medium. As mentioned in the introduction to the experimental part of this work it was desired that the following elements be incorporated in the medium: C, N, O, P, K, Ca, Mg, S, and Cl. From the experimental work cited above the following, seemed most suitable.

C, and O as sucrose (10 per cent)
N, and Cl as NH_4Cl (0.12 per cent)
K, and P as K_2HPO_4 (0 05 per cent)

Bearing in mind the work of Loew (1899), that Ca and Mg salts were necessary for nucleus production, Ca was added in the form of $CaCl_2$, Mg in the form of $MgSO_4$; and in the concentrations which appeared to be universal with earlier workers, namely, $CaCl_2$ 0.01 per cent and $MgSO_4$ 0.02 per cent. With the addition of these two salts all the essential elements were incorporated in the medium.

Formula of the new medium (called Medium No. 1)

Sucrose .... 100.0 grams
NH_4Cl .... 1.2 grams
K_2HPO_4 .... 0.5 gram
$CaCl_2$ .... 0.1 gram
$MgSO_4$ .... 0.2 grams
Distilled water.. 1000 cc.

This medium was found to form only a slight precipitate. It was sterilized for twenty minutes at 15 pounds.

Observations were made on the growth of the yeasts in medium no. 1 for eighteen more weeks. *Pichia farinosus, Saccharomyces albus, Saccharomyces cerevisiae,* and *Saccharomyces ellipsoideus* displayed abundant growth; while *Saccharomyces marxianus* and *Saccharomyces Pastorianus* showed a moderate growth. The tubes were well shaken before a count was made, which aerated the cultures to some extent and aided in multiplication.

Pichia farinosus formed a pellicle growth and climbed the walls of the tubes. After several days incubation the growth was heavy enough to hang down into the medium. The other yeasts grew so as to produce a uniform clouding. All of the yeasts produced a sediment. *Saccharomyces cerevisiae* and *ellipsoideus* liberated gas.

PURIFIED SUCROSE IN A SYNTHETIC NUTRIENT MEDIUM

A large number of synthetic nutrient solutions (inorganic salt and sugar media) for the cultivation of yeasts have been proposed. In a fair percentage of these, sucrose is employed as the source of carbon.

In these various synthetic media the sugar is the only constituent whose purity has been questioned, being thought by many to contain growth promoting substances as impurities.

The object of this part of the investigation was to determine if any growth promoting substance which might be contained in sucrose as an impurity could be removed by alcoholic extraction. The consensus of opinion was that "bios" was present in the sugar and would be soluble in 80 per cent ethyl alcohol.

The extraction of "bios" from cane sugar was attempted by Willaman and Olson (1923). The sugar was extracted with 80 per cent ethyl alcohol by repeated precipitation from a concentrated water solution since "bios" was considered soluble in this concentration of alcohol. Following experiments in which this extracted sugar was employed, they concluded that normal growth of yeast was impossible without "bios" and that, up to an optimum concentration, the rate of growth and total amount of growth were roughly proportional to the amount of "bios" available. They pointed out that over sixty compounds of nitrogen have been tested and found not to be "bios."

Merck's (Highest Purity) Saccharose was employed throughout the experiment. The method of purification was as follows: A supersaturated solution of sucrose in water was first made by dissolving an excess of sucrose in hot water. When the solution was cool the saturated syrupy solution was poured off and its volume determined. To this solution enough 95 per cent ethyl

alcohol was added so that the resulting solution contained 80 per cent alcohol. To do this it was necessary to add 275 cc. of 95 per cent ethyl alcohol for every 50 cc. of saturated sugar solution present. Allowance was made for the 5 per cent water in the alcohol. The solution was then set aside and the sucrose allowed to crystallize. As a rule it was unnecessary to seed the solution. After the crystallization was complete (twenty-four to thirty-six hours) the sucrose was filtered off, washed with 80 per cent alcohol, dried, powdered, and used.

Since the formula of Medium No. 1 had not been devised at the time this work was being carried on Fulmer and Nelson's Medium E was used to test the properties of the extracted sugar and the residue.

Formula of Fulmer and Nelson's Medium E. Ammonium chloride, 0.188 gram; calcium chloride, 0.10 gram; di-potassium hydrogen phosphate, 0.10 gram; calcium carbonate, 0.040 gram; sucrose, 10.0 gram; water (distilled) 100 cc.

In preparing the medium both raw (not extracted) and extracted sucrose were used. The same procedure and technique was employed as was described earlier in this paper. The medium was tubed in 10 cc. quantities as needed, and inoculated with water suspensions of the six yeasts used in the work on the salt requirements of yeasts, and incubated at 30°C. The counts were determined, as before, with a haemacytometer.

The "bios" factor, if present, being soluble in 80 per cent ethyl alcohol, remained in the filtrate. To determine this a portion of one of the filtrates was evaporated to dryness on a water bath and the residue examined. A 10 per cent water solution of this was added to both raw and extracted sugar media in varying quantities so that 0.01, 0.02, 0.03 . . . . 0.10 per cent mixtures were formed.

It was found that no growth promoting substance, soluble in 80 per cent ethyl alcohol was demonstrated in the sucrose by the methods used. The experiment was continued long enough, and sufficient transfers were made so that any growth stimulating substance present in the initial transfer was eliminated. The initial inoculations into the four types of media: (a) raw

sugar medium, (b) extracted sugar medium, (c) raw sugar medium containing water solution of residue, and (d) extracted sugar medium containing water solution of residue—were made from the same emulsion; consequently these cultures were started with the same number of cells for each yeast employed. The counts, as determined by a haemacytometer, were practically the same (several million cells per cubic centimeter) for each yeast in the four types of media. The small differences were within the range of experimental error.

EFFECT OF AERATION ON YEAST GROWTH

Another factor in yeast growth and multiplication which has aroused the interest of several investigators is that of aeration.

Hansen (1902) pointed out the necessity of air or oxygen in media for sporulation of yeasts. Slator (1921) found that air aided yeast growth owing to the effect it had in lessening the supersaturation of the medium with CO_2. The early stages of growth of quiescent yeast did not require air.

Tanner and Millhouse (1925) found that aeration had a decided effect, the multiplication rate being many times greater in the aerated than in the unaerated medium.

The object of this phase of the experimental work was to determine whether or not aeration had any influence on the multiplication of yeasts.

Compressed air, used for the aeration, was sterilized by passing it through a train of two bottles packed with cotton. The bottles were about 10 cm. in diameter and 25 cm. high. The air was then carried by means of glass and rubber tubing to a "Y" tube; which was connected to two culture bottles. These culture bottles were fitted with two-hole rubber stoppers. They were wide mouth bottles about 7 cm. in diameter and 18 cm. high. The air was carried to the bottom of the bottles by means of Folin tubes (used to break up the air bubbles). The air left the culture bottles through short delivery tubes to the ends of which were connected rubber tubes plugged with cotton.

The entire apparatus was placed in a 30°C. incubator, and the air was brought in through a small hole in the side of the

incubator. The delivery tubes and the bottles containing the cotton were autoclaved before the experiment was started. The medium was sterilized in the culture bottles along with the stoppers, Folin tubes, and delivery tubes.

Five hundred cubic centimeters of Medium No. 1 were placed in each culture bottle and sterilized as described above. The bottles were inoculated with one cc. of a seven-day culture of the yeast. The yeasts used had been cultured in this same medium for at least seven weeks. Pure cultures of the following yeasts were used: *Saccharomyces cerevisiae*, *Saccharomyces ellipsoideus*, *Saccharomyces albus*, *Pichia farinosus*, *Saccharomyces marxianus*, and *Saccharomyces Pastorianus*.

For comparison, unaerated culture bottles were placed in the incubator along with those that were aerated. Haemacytometer counts were made every day and curves plotted.

In accordance with the results of Slator (1921), and Tanner and Millhouse (1926) aeration was found to have a decided effect upon the growth of the yeasts studied.

Saccharomyces cerevisiae responded very slightly to aeration; the unaerated count reached a maximum of 10.3 millions of cells per cubic centimeter while the aerated count reached a maximum of only 13.7 millions. The other yeasts, however, responded to aeration to such an extent that the aerated maximum counts were from 3 to 15 times the unaerated maximum counts. The effect of aeration was best noted with *Pichia farinosus;* the maximum unaerated and aerated counts were respectively 24.0 and 320.0 millions of cells per cc. This yeast was the only one, which, when not agitated, formed a pellicle. This suggested some correlation between yeasts which when not agitated form pellicles and those which respond to aeration.

The unaerated cultures were not entirely without aeration since they had to be shaken each day before the counts were made. If these counts could have been made without this small amount of aeration, due to the shaking, there probably would have been even greater differences between the counts of the aerated and the unaerated cultures.

As stated before, the removal of CO_2 by the aeration seems

to be the best explanation of the great increase in multiplication. It was also noticed that with the aerated cultures the clumps were smaller and the buds were broken off; this might have aided in a more rapid multiplication.

STAINING LIVE AND DEAD YEAST CELLS

Many methods have been proposed for the differentiation of living and dead bacteria and yeast cells. According to Wehmer, a 0.5 per cent methylene blue solution stained dead yeast cells indigo blue and left the living cells colorless. Schlichting and Winther (1910) investigated two stains; methylene blue (1:1000) and sodium sulfoindigotate (1–30). Of the two staining media employed they preferred the solution of sodium sulfoindigotate (Indigocarmine) in the dilution of 1:30.

Fraser (1920) employed a larger group of stains: Grubler's methylene blue, Merck's methylene blue, Kahlbaum's methylene blue 6B extra, fuchsin, congo red, erythrosin, safranin (water soluble), gentian violet, and methyl green. The action of these stains was determined on "boiled" and "live" baker's yeast, and on yeast killed by mixing with 0.25 per cent phenol for three hours. They found no differences in staining properties of the yeasts killed by boiling, and with phenol. Erythrosin (0.5 per cent) and Kahlbaum's methylene blue 6B extra (about 0.1 per cent) proved the best. The erythrosin gave a sharp distinction between live (stained red) and dead (colorless) cells in three minutes, and the methylene blue, in one minute after the addition of the dye.

The following stains in the concentrations noted were used in this investigation: Congo Red, Eosin, Erythrosin, Methyl Green, Neutral Red, Safranin (0.5 gram 100 cc. water); Gentian Violet, 0.4 gram per 100 cc. water; Indigo Carmine, 1.0 gram per 30 cc. water; Methylene Blue, 1.0 gram per 200 cc. water; and Methylene Blue, 1.0 gram per 1000 cc. water.

The procedure used in determining the efficiency of a stain to differentiate between living and dead yeast cells was very simple. Young cultures of *Saccharomyces cerevisiae* and *Saccharomyces ellipsoideus* were used as a source of living cells. In

these young cultures only a small per cent (in many cases less than 1 per cent) of the cells were found to be dead. Young cultures killed by boiling for a few minutes or by mixing with 0.50 per cent phenol for a few hours acted as a source of dead cells. In staining, either dead or living cells, equal volumes of stain and yeast suspension were mixed and allowed to stand for one minute. A wet mount was then made and examined by a magnification of about 500. After it was determined qualitatively whether or not a stain had the ability to differentiate between living and dead cells (dead cells being stained while the living cells were not stained) a quantitative determination was made, as follows: A water suspension of a young yeast culture was divided into two portions; one portion was killed by one of the methods described above. In this way two suspensions having approximately the same count per cubic centimeter were obtained. Equal volumes of the two suspensions were mixed and stained as directed above. When examined, the number of stained (dead) cells should be approximately equal to the number of unstained (living) cells. A number of fields were counted and an average determined.

The experiment was repeated, using, as a source of dead cells, cells that had died from the lack of food material or accumulation of metabolic products.

No difference was found in the response of dead cells to the stains when killed by heat or with phenol, or when they died from lack of food material or "old age."

Congo Red. The dead cells were stained only a faint pink and it was difficult to distinguish living cells from dead cells.

Eosin. The dead cells were stained a faint pink, while the living cells were colorless. As compared to Erythrosin it was more difficult to distinguish dead cells from living cells. A large number of fields of a stained suspension of living and dead cells were counted and the average number of dead and living cells per field was determined. The average number of dead cells per field was found to be 14.1, and for living cells 15.6.

Erythrosin. The dead cells were stained a deep pink while the living cells were colorless; which made differentiation easy.

The average number of dead cells per field for a large number of fields was found to be 23.1, and for living cells 26.6.

Gentian Violet. In the concentration employed this stain proved to be unsatisfactory since the living as well as the dead cells were stained.

Indigo Carmine (sodium sulfo-indigotate). This stain also proved to be unsatisfactory; the dead cells were not stained after being in contact with the dye for as long a period as thirty minutes.

Methylene Blue (1:200). Methylene blue of this concentration was not as satisfactory as the 1:1000 concentration. The living cells were slightly tinged with blue which was not very satisfactory for differentiation.

Methylene Blue (1:1000). The living cells were not stained, while the dead cells were stained blue. Although differentiation of dead cells from living cells was possible with methylene blue 1:1000, it was not as satisfactory as Erythrosin or Eosin. The average number of dead cells per field for a large number of fields was found to be 24.8, and for living cells 25.1.

Methyl Green. The dead cells were stained a blue-green while the living cells were only tinged with green. Although differentiation was possible the stain was not as satisfactory as Erythrosin. The average number of dead cells per field for a large number of fields was 33.3, and for living cells 34.7.

Neutral Red. Nearly all the cells were stained a deep pink or at least tinged with pink. This made differentiation nearly impossible.

Safranin. This stain was only a trifle more efficient than neutral red. The dead cells were stained red and the living cells tinged with pink, which placed the stain in the group which were considered as unsatisfactory.

GENERAL SUMMARY

The synthetic medium proposed by Robertson and Davis (1923) was found to be similar in composition, in many respects, to those media proposed by Fermi, Nageli (1881), and Fulmer and Nelson (1923). Contrary to the findings of Robertson

(1924) the medium of Robertson and Davis was found to support the growth and continuous multiplication of a variety of yeasts. Growth and continuous multiplication of the same group of yeasts was also obtained when the sodium chloride was omitted from the medium. Taking the entire set of cultures as a whole the presence or absence of sodium chloride in the medium, in the concentration used, appeared to have no decided effect, as suggested by Mitra (1917).

With regard to the construction of a synthetic nutrient medium it was desired to incorporate the elements C, N, O, P, K, Ca, Mg, S, and Cl in the medium. These elements were added in the form of the following compounds: NH_4Cl, K_2HPO_4, $CaCl_2$, $MgSO_4$, and sucrose. After the optimum concentration of each compound (except sucrose) was determined and the medium prepared it was found that six pure species of yeasts were able to multiply abundantly, when cultured according to the technique described. The fact that this medium forms no precipitate and yet contains a buffer makes it very satisfactory as a basal medium.

As mentioned earlier, the only compound whose purity (considering "bios" as an impurity) could be questioned was the sucrose. However, sucrose, recrystallized according to the method described, when compared to ordinary sucrose, produced no noticeable effect on the continuous multiplication and growth of the six pure species of yeasts when cultured in Fulmer and Nelson's Medium E. To approach the question from another angle the filtrates, resulting from the recrystallization of the sucrose from 80 per cent ethyl alcohol, were examined. When these filtrates were concentrated and added to the medium above (prepared from ordinary and recrystallized sucrose) they were found to produce no stimulating effect on the multiplication of the yeasts used. From the above it appears that no growth promoting substance soluble in 80 per cent ethyl alcohol was demonstrated in the sucrose employed. Also, it appears that multiplication can be obtained with large seedings in a synthetic "bios" free medium providing the medium is one in which the yeasts can avail themselves of the foods present. It must not

be forgotten that there are factors other than chemical factors that influence growth. Aeration, a physical factor, was the one studied. The response of the yeasts to aeration was very striking. Counts of several hundred millions of cells per cubic centimeter were obtained in the aerated flasks in contrast to only 10 to 30 millions of cells per cubic centimeter in the unaerated flasks. This increase is thought to be due to the removal of CO_2 from the medium and also to the breaking off of the buds due to the agitation. The results also suggested a possible correlation between top yeasts and their response to aeration.

Another problem of a different nature from those discussed above, but included in this investigation, is that of staining live and dead yeast cells. An attempt was made to compare the ability of a number of stains to differentiate between living and dead cells, (dead cells being stained, living cells unstained). Of the stains employed (congo red, eosin, erythrosin, gentian violet, indigo carmine, methylene blue, methyl green, neutral red, and safranin), erythrosin (0.25 per cent aqueous solution when mixed with the yeasts suspension) was found to be the most efficient and desirable. Eosin and methylene blue (0.25 per cent and 1:2000 by weight respectively when mixed with the yeast suspension) were found to be efficient but not as satisfactory as erythrosin. No difference was found in the response of dead cells to the stains when killed by heat or with phenol, or when they died from lack of food material or "old age."

CONCLUSIONS

1. Robertson and Davis' medium was found to support the continuous multiplication of 26 yeasts.

2. Robertson and Davis' medium without sodium chloride was found to support the continuous multiplication of 24 yeasts.

3. Taking the entire set of yeasts as a whole, the presence or absence of sodium chloride in the medium, in the concentration used, appeared to have no decided effect. In the case of *Saccharomyces cerevisiae* the presence of sodium chloride (5.0 grams per liter) appeared to have a harmful effect.

4. Six pure species of yeasts were found to multiply either moderately or abundantly in Medium No. 1, when cultured according to the technique described.

5. Multiplication can be obtained with large seedings in a synthetic "bios" free medium providing the medium is one in which the yeasts can avail themselves of the foods present.

6. Sucrose, recrystallized according to the method described, when compared to ordinary sucrose, produced no noticeable effect on the continuous multiplication of six pure species of yeasts when cultured in Fulmer and Nelson's Medium according to the technique described.

7. Filtrates, resulting from the recrystallization of the sucrose from 80 per cent ethyl alcohol, when concentrated and added to Fulmer and Nelson's Medium E (prepared by using ordinary and recrystallized sucrose), produced no stimulating effect on the multiplication of the yeasts used.

8. No growth promoting substance soluble in 80 per cent ethyl alcohol was found in the sucrose employed.

9. Aeration increased the multiplication of the yeasts employed when cultured according to the technique described.

10. The removal of CO_2 by the aeration seems to be the best explanation of the increase in the multiplication.

11. The results suggest a possible correlation between top yeast and response to aeration.

12. Living and dead yeast cells can be differentiated by staining methods.

13. Erythrosin (0.25 per cent aqueous solution when mixed with yeast suspension) was found to be the most efficient and desirable of the ten stains examined for differentiating living and dead yeast cells.

14. Eosin (0.25 per cent aqueous solution when mixed with the yeast suspension) and Methylene blue (1:2000 aqueous solution when mixed with the yeast suspension) were found to be efficient but not as satisfactory as Erythrosin.

15. No difference was found in the response of dead cells to the stains when killed by heat or with phenol, or when they died from lack of food material or "old age."

REFERENCES

FRASER, C. G. 1920 The action of methylene blue and certain other dyes on living and dead yeasts. Jour. Phys. Chem., **24**, 741–748.

FULMER, E. I. 1925 Effect of ammonium salts on the swelling of colloids and the growth of yeasts at various temperatures. 2nd Colloid Symposium Monograph, pp. 204–208. Reviewed in J. C. S., **128** (1925), 1508.

HANSEN, E. C. 1902 Comparative investigations on the conditions of growth and reproduction of yeast. Comp. Rend. Trav. de Carlsberg, **5**, 68–107.

MITRA, S. K. 1917 Toxic and antagonistic effects of salts on wine yeasts. Pub. Agr. Sci., **15**, 62–102. Reviewed in Chem. Abs., **12** (1918), 513.

NELSON, V. E., FULMER, E. I., AND CESSNA, RUTH 1921 The nutritional requirements of yeast. III. The synthesis of water soluble B by yeast. Jour. Biol. Chem., **46**, 77–81.

LOEW, O. 1899–1900 The physiological rôle of mineral nutrients. U. S. D. A. Div. of Vegetable Physiology and Pathology Bull. 18, pp. 60. E. S. R. **11**, 1008.

ROBERTSON, R. C. 1924 Food accessory factors (vitamins) in bacterial growth. IX. Growth of several common bacteria in a synthetic medium and relation of substances formed by them to growth of yeast. Jour. Inf. Dis., **35**, 311–314.

ROBERTSON, R. C., AND DAVIS, D. J. 1923 Food accessory factors (vitamins) in bacterial growth. VII. Observations on the ultimate source of accessory growth substances for yeast. Jour. Inf. Dis., **32**, 153–158.

SCHLICTING, E., AND WINTHER, H. 1910 A criticism of staining methods for determining the dead cells of yeasts. 7th International Congress of Applied Chemists, Sec. VI B.

SLATOR, A. 1921 Yeast crops and the factors which determine them. Jour. Chem. Soc., **119**, 115–131.

TANNER, F. W. 1924 The bios question. Chem. Rev., **1**, 397–472.

TANNER, F. W. AND MILLHOUSE, LOUISE 1926 Observations on the growth of yeasts in pure nutrient solutions. Proc. Soc. Exp. Biol. and Med., **23**, 447–449.

WILDIERS, E. 1901 Une nouvelle substance indespensible au development de la levure. La Cellule, **18**, 313–331.

WILLAMAN, J. J., AND OLSON, A. G. 1923 The bios requirement of bakers' yeast. Jour. Biol. Chem., **55**, 815–836.

VITAMIN EFFECTS IN THE PHYSIOLOGY OF MICRO-ORGANISMS

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No sooner had vitamins been discovered to be essential in the growth and reproduction of certain of the higher animals than similar claims were put forth in regard to their importance in the physiology of microörganisms. Confusion as to the identity of bios and vitamin B led to further unjustifiable extensions of the meaning of the term vitamin until substances acting as growth or reproductive stimulants were included in the category of vitamins, although wholly unnecessary for the processes of growth and reproduction themselves. In fact as Fulmer (1922) pointed out it has been attempted on the basis of just such stimulation to ascribe entirely new properties to vitamins in direct conflict with the only adequate test now known—the effect on the growth and reproduction of a suitable animal.

Substances of the nature of vitamin B have been claimed to be essential or markedly stimulating to the growth and reproduction of various microörganisms. Only a few of the more pertinent references will be cited here and the reader is referred to Tanner's review of the bios question for a comprehensive resumé (1925).

Linossier (1919, 1920) reported that *Oöspora lactis* could grow in synthetic media but that it was sensitive to the addition of vitamins. The addition of vitamin extracts markedly increased the crop. Sammartino (1921) reported that both rapidity of reproduction and rapidity of fermentation of *Saccharomyces cerevisiae* are affected by vitamins.

Funk and Friedman (1922) found for both yeasts and bacteria growth stimulating substances which they claimed to be of a vitamin nature, present in beef, pepton, beef heart infusions and

autolyzed brewer's yeast. Suzuki and Suzuki (1923) separated from vitamin B by means of aluminum cream a substance promoting bacterial growth, which they called vitamin D and stated that it was a decomposition product of vitamin B. Broadhurst (1921) reported remarkable revivification of old bacterial cultures by use of an aqueous extract of the navy bean. She attributed the results to vitamins.

Itano (1923) in a study of the influence of vitamin B upon the rate of growth of *Azotobacter chroococcum* concluded that it exerted a marked stimulating influence on reproduction and nitrogen fixation. Referring to vitamin B concentrate and nucleic acid Itano concluded that "it seems to be apparent that these two substances exert an accessory influence other than the supply of a very small quantity of food substance." He believed his experimental data indicated plainly that the addition of vitamin B to the medium had a stimulating influence on *Azotobacter chroococcum*.

Sanborn (1926) in a recent article concluded that "the essential food factor represented by vitamin B (?) exerts a stimulating effect upon the growth and physiological efficiency of *C.* (*Cellulomonas*) *folia*." In the work of Itano and of Sanborn a vitamin concentrate was added to the medium to supply vitamin B.

The numerous investigations purporting to indicate a stimulative effect of vitamin B on bacterial metabolism show a general disregard of the effect of small and easily available quantities of food present during the lag phase on the bacterial numbers occurring during the phase of logarithmic reproduction. A similar disregard of the possible effect of the vitamin synthesized by the microörganism is manifested.

In a former communication (1925) it was shown that numerous species of bacteria, yeasts and fungi may be continuously cultured in wholly synthetic media employing inorganic salts and either methose, or synthetic succinic acid, as sources of energy. In the present paper it is shown that the addition of vitamin B to the medium does not stimulate reproduction of certain nitrogen fixing organisms, particularly *Azotobacter chroococcum*. It is shown that the addition of a vitamin B *concentrate* may afford a

transient stimulation which is not of a type correctly ascribed to vitamins and is incidentally the result of circumstances in which vitamin B plays no essential part. It is shown that stimulation due to a small quantity of food substance other than vitamin B may be differentiated from effects ascribable to vitamins by determining the rates of multiplication of an organism.

<div align="center">EXPERIMENTAL</div>

Effect of vitamin B on the rate of multiplication of Azotobacter chroococcum and Rhizobium leguminosarum

The vitamin B concentrate used was prepared by the Harris Laboratories. The preparation was advertised as containing vitamin B in concentrated form (Osborne and Wakeman, fraction 2). Rat tests indicated that the material was rich in vitamin B. Its nitrogen content by the Kjeldahl method was 0.75 mgm. per gram of concentrate. It was tested for its action on the rate of multiplication of microörganisms by adding to Ashby solution of pH 7.2. This medium contained only chemically pure salts and sucrose. As a precaution against the possible presence of vitamin B, the sucrose was extracted continuously for seventy-two hours with hot 95 per cent alcohol. A 1 per cent solution of the concentrate was prepared and varying amounts added to double strength Ashby solution. Sterile distilled water was then added to volume. The medium was tested for sterility before use. Equal quantities of a twenty-four to forty-eight hour culture of *Azotobacter chroococcum* or *Rhizobium leguminosarum* were added as inoculum.

All culture flasks in an experiment were connected by means of a manifold to a vacuum line and the number of bubbles of air passing through controlled by means of screw clamps. A current of air sufficient to maintain a circulation of the medium was employed. Cotton plugs in the inlets and outlets prevented contamination; an acid tower prevented absorption of atmospheric ammonia by the cultures. Reproduction of Azotobacter and Rhizobium is markedly stimulated by aeration due to a better distribution of oxygen and nitrogen throughout the

medium. The range of growth in the medium is greatly extended, that is, growth and reproduction occur throughout the medium and not simply near the surface as occurs in unaerated liquid cultures.

Plate counts were abandoned since any strain of Azotobacter or Rhizobium forms some gum and plate counts were found unreliable. Direct counts were made by use of a Helber counting chamber.

Stimulation of bacterial multiplication may best be determined by comparing velocity coefficients of the rate of multiplication. This is most conveniently performed for the logarithmic phase. Our classification of the life phases in a bacterial culture is that suggested by Buchanan (1918). During the logarithmic phase the rate of multiplication is constant. The velocity coefficient (k) is determined by the relationship

$$k = \frac{\log b - \log B}{t}$$

in which k is the velocity coefficient, i.e., the rate of reproduction per cell, t is time in hours, b is the number of bacteria present after time t, and B the number of bacteria present when t is equal to 0. Graphical representation of results may be accomplished by plotting logarithms of the numbers of bacteria as ordinates against times in hours as abscissae. Since the rate of multiplication during the logarithmic phase remains constant, the curve during this period is represented by a straight line. The greater the slope of this line the greater the rate of multiplication. We may, therefore, compare graphically the rates of multiplication of two cultures during the "logarithmic" phase by comparing the slopes of these lines. Since a straight line represents the rate of multiplication, coefficients may be determined for any interval of time during this phase. However, the entire line was employed to determine velocity coefficients because it was found that more accurate results were obtained in this manner.

Experimental determination of a mathematical expression of the degree of stimulation occurring during the lag phase is more difficult since there is an acceleration of the rate of multiplication

per cell which approaches the constant of the logarithmic phase. A time measurement of lag may be of value in these experiments since all cultures were started with equivalent inoculations.

Table 1 gives the results of one of numerous experiments and affords data which are subject to analysis. In a 1:10,000 concentration, the vitamin *concentrate* evidently induces a definite increase in the number of organisms present during the early

TABLE 1

Effect of vitamin B on the rate of reproduction of Azotobacter

FLASK NUMBER	TREATMENT (ASHBY MEDIUM)	ORGANISMS, MILLIONS PER CUBIC CENTIMETER AFTER HOURS AT 25°C.							k
		0 hours	6 hours	12 hours	18 hours	30 hours	42 hours	54 hours	
1	Vitamin concentrate 1:1,000	2.0	4.0	8.3	16.0	58	230	916	0.872
2	Vitamin concentrate 1:10,000	2.0	3.5	6.4	14.0	46	190	750	0.881
3	Vitamin concentrate 1:1,000,000	2.0	2.2	3.0	4.6	19	78	300	0.872
4	Alcohol extract equivalent to 1,100,000 concentrate	2.0	2.2	2.9	4.9	18	84	300	0.860
5	Extracted concentrate 1:1,000	2.0	3.9	7.6	14.0	50	210	860	0.915
6	Plain Ashby control	2 0	2.3	3.4	6.5	23	91	320	0.835

stages of growth. This holds for both *Azotobacter chroococcum* and *Rhizobium leguminosarum* (table 1).

In figure 1 are shown for more critical analysis curves for the multiplication of *Azotobacter chroococcum*. Logarithms of the numbers of bacteria are plotted as ordinates, times in hours are plotted as abscissae. An analysis of the curves indicates the nature of the apparent stimulation. It is shown that stimulation due to small quantities of food substances other than vitamin B may be differentiated from effects ascribable to vitamins by determining rates of multiplication of the organisms. Cultures

to which the vitamin B concentrate was added showed a greater average acceleration—a progressively shorter average generation time—during the period of lag than did the control organisms forced to fix atmospheric nitrogen. Lag periods of ten to fifteen hours were common in the control flasks. On the other hand the addition of vitamin B concentrate in 1:1000 concentration re-
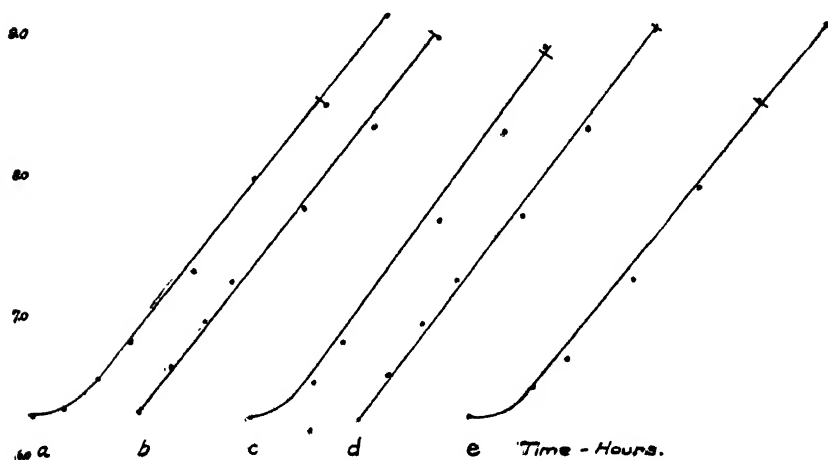
FIG. 1

A. Culture in Ashby medium (control).
B. Culture in Ashby medium plus 1:10,000 vitamin B concentrate.
C. Culture in Ashby medium plus 1:1,000,000 vitamin B concentrate.
D. Culture in Ashby medium plus 1:10,000 alcohol extracted vitamin B concentrate.
E. Culture in Ashby medium plus alcohol extract of vitamin B concentrate equivalent to 1:10,000 concentrate.

duced the lag to a period of an hour or less. It is difficult to say whether the initial stationary phase is shortened by the vitamin concentrate or whether the rate of reproduction is simply greater during the lag phase. It has seemed by using old cultures of Azotobacter (i.e., cells capable of showing an extended initial stationary phase) that both a reduction of the initial period and

an increased rate of reproduction are caused by the addition of the vitamin concentrate.

Following this initial acceleration in the cultures containing the vitamin concentrate the curves become parallel and the average rates of reproduction—the average generation times in the control and vitamin cultures—are equal. That is, the cells in the original inoculum and their progeny for several generations show the stimulative action of the concentrate while cells in following generations show no such effect.

This is clearly not a vitamin effect. There is no reason to assume that the first generations would be stimulated by vitamin B while the later generations would not. A vitamin effect should be indicated by an increase in the rate of multiplication extending throughout the entire range of reproduction—at least through the lag phase and logarithmic phase. The curve during the logarithmic phase for cells receiving a vitamin stimulation would be expected constantly to ascend more rapidly than the control curve. This is apparently not the case.

Against these conclusions it might be claimed that the vitamin is used up during the lag phase and that the stimulation due to the vitamin must necessarily cease therefore. Furthermore, it is conceivable that destruction of the vitamin may occur as the result of the action of bacterial products of metabolism. Investigators claiming to have shown vitamin stimulation have tacitly assumed no such induced unavailability of the vitamin, since none of them have restricted their claims to the lag phase. In fact the nature of their experiments is assurance that their results were determined from multiplication occurring during advanced life phases. In view of the fact that a concentrated form of vitamin material was added, it is hardly conceivable that a few million bacteria could possibly use up all of the vitamin. Furthermore, we know that old cultures of Azotobacter have vitamin B available for the growth of rats. Our results with alcohol extracts of vitamin concentrate and extracted concentrate show a definite correlation of the rate of multiplication with the quantity of nitrogenous material and not with vitamin content. This point will again be considered.

If on the other hand we are dealing with a nutritional stimulation due to the presence of a small quantity of an easily available source of some essential element such as nitrogen or due to an easily available source of energy, such stimulation would be apparent only so long as the source of supply lasted. Therefore, we may determine experimentally, velocity coefficients of the rate of multiplication during the logarithmic phases of cultures of organisms with and without vitamin B added to the medium. If the addition stimulates reproduction, the coefficient, determined in the presence of vitamin B will be greater. The only other alternative is proof of the destruction or induced unavailability of the vitamin.

The utilization of a very small quantity of food material other than vitamin B by the organisms constituting the original inoculum, resulting in a greater acceleration than is shown by organisms forced to fix atmospheric nitrogen, is important in determinations purporting to show stimulation ascribable to vitamins. This point has been generally disregarded.

An hypothetical case may serve as an illustration. Culture tubes A and B containing Ashby's medium are inoculated with equal numbers of Azotobacter cells. Tube B receives an addition of a small quantity of a substance containing readily available nitrogen. It is necessary for the organisms in tube A to obtain their nitrogen from the atmosphere (a less available source than is afforded by the substance added to tube B). As a result, the organisms planted in tube B multiply more rapidly and are, let it be assumed, ten times as numerous per unit volume as in tube A at the time each enters the logarithmic phase. At this time the source of nitrogen available in tube B as the result of the addition of the nitrogenous material to the medium, becomes exhausted. Both cultures must now obtain their nitrogen from the atmosphere. Disregarding any environmental adaptations of the organisms and recalling that multiplication is geometric, we shall find (at comparable times during the logarithmic phases in the cultures) always ten times as many organisms in tube B as are in tube A. In fact, this is just the result to be expected if the rates of multiplication per cell are equal. Resort

to an hypothesis of vitamin stimulation is not only superfluous but involves an erroneous interpretation of results.

An experimental demonstration of the above hypothesis is shown in figure 1. It is seen that after an initial stimulation of the rate of multiplication in the vitamin tubes, all rates are later equivalent (slopes parallel) although there are many more organisms in the vitamin B culture per cubic centimeter than in the control. The rate of multiplication per cell (k) for organisms having access to vitamin B in 1:10,000 concentration is 0.881, for controls 0.835 (table 1). At 1:10,000 dilution the concentrate contains sufficient easily available nutritional matter of a nature other than vitamin B (i.e., nitrogen) to afford these organisms an initial stimulation that carries them into the phase of logarithmic reproduction in the culture. The effect is then exhausted and stimulation ceases. The effect is purely that of providing the organisms with a better medium than is afforded by Ashby medium.

Itano (1923) and Sanborn (1926) disregard the significance of the food substances other than vitamin B in the concentrate. Itano states that fraction 2 contains 7.5 per cent nitrogen, the same as found by us but we cannot agree that this quantity is insignificant, since in a 1:10,000 concentration there would be present in 100 cc. of the medium used 0.75 mgm. of nitrogen.[1] Assuming a bacterial cell to be 85 per cent water and 15 per cent solid matter, 90 per cent of the solid matter to be protein, and 16 per cent of protein to be nitrogen, 0.75 mgm. of nitrogen would suffice for approximately 17 billions of bacteria of average size and weight (0.000,000,002 mgm.) if all the nitrogen were available for cell synthesis.

To test this assumption *Bacterium coli* was inoculated into a medium consisting of Harris vitamin B concentrate and distilled water, and into the same medium to which had been added 1 per cent glucose as a source of energy, and 0.2 per cent dipotassium phosphate. That vitamin B concentrate supplies all the elemental and energy needs of an organism is obvious from

[1] Itano states that in a 1:10,000 concentration of vitamin B concentrate which was used by him in 100 cc. quantities of medium there would be present .0075 mgm. of nitrogen. This is apparently an erroneous conclusion.

table 2.   In a medium consisting only of 1:10,000 concentration of vitamin B concentrate *Bact. coli* was afforded all the elemental matter and energy requisite for the development of at least 40 millions of bacteria per cubic centimeter.   If a source of energy were supplied as is the case in experiments using Ashby's medium at least 63 millions of bacteria per cubic centimeter developed. Itano and Sanborn disregard the effect of that part of the concentrate other than vitamin B and comprising the great bulk of the material and ascribe the stimulation to vitamin B.

TABLE 2

Multiplication of Bacterium coli in medium containing vitamin B concentrate as source of nutriment

FLASK NUMBER	MEDIUM 1 PER CENT DEXTROSE, 0.2 PER CENT K₂HPO₄ PLUS CONCENTRATE	ORGANISMS AFTER HOURS AT 25°C. (MILLIONS PER CUBIC CENTIMETER)		
		0 hours	24 hours	48 hours
1	1:1,000	0.25	35.0	740
2	1:10,000	0.26	13.0	63
3	1:100,000	0.24	4.0	18
4	1:1,000,000	0.25	1.1	3
5	1:1,000 concentrate, as sole medium	0.29	27	480
6	1:10,000 concentrate as sole medium	0.27	11.0	40
7	1:100,000 concentrate as sole medium	0.26	0.3	11.0
8	1:1,000,000 concentrate as sole medium	0.24	0.03	0.2
9	Distilled water only	0.27	0.20	0.2

Inasmuch as vitamin B is soluble in alcohol, if the vitamin itself is effective in stimulating growth, a very small quantity of an alcoholic extract of the concentrate should be adequate to demonstrate the effect.   The following experiments reveal no vitamin stimulation when such extracts were substituted for aqueous solutions of the vitamin B concentrate.

A weighed quantity of concentrate was continuously extracted with hot 95 per cent alcohol for twenty-four hours when the alcohol was evaporated *in vacuo* until 0.1 cc. represented the extract from 0.01 gram of concentrate.   This material contained

small amounts of alcohol soluble nitrogenous compounds, approximately 0.1 mgm. per 0.1 cc. of extract which represented 0.01 gram of concentrate. This quantity when added to a liter of medium represented a concentration of 1:10,000 of concentrate. No stimulation due to food substance was detectable in this concentration. It is obvious from the curves and table 1 that no stimulation ascribable to vitamins was present. It is reasonable to expect a vitamin effect to be apparent under these conditions if such an effect exists.

Furthermore, it is reasonable to expect, if vitamin exerts no stimulation of bacterial multiplication, that alcohol extracted concentrate would exert the same type of stimulation as that observed in the case of unextracted concentrate. It is possible that the alcohol used in extracting would remove desirable proteins, carbohydrates or other substances along with vitamin B. A very small quantity of material is removed but this removal did not detectably influence the results of the experiments. When comparing the extracted and unextracted material, the nitrogen contents were made equivalent. This required 0.0125 gram of extracted per 0.01 gram of unextracted material.

In table 1 is shown the stimulation resulting from the addition of vitamin concentrate continuously extracted for three weeks with hot 95 per cent alcohol. The same stimulation occurs as with the unextracted concentrate.

Waksman and Skinner (1926) have recently demonstrated the stimulation afforded to cellulose fermenters by the addition of available nitrogen to the medium. They found that the amount of cellulose which is decomposed in the soil under aerobic conditions is dependent upon the amount of available nitrogen and that aerobic decomposition of cellulose sets in very early if available nitrogen is present. Niklewski (1912), Charpentier (1921), Barthel and Bengston (1924) and Waksman and Heukelekian (1924), all found that the decomposition of cellulose in the soil is largely controlled by the amount of available nitrogen. Sanborn (1926) apparently ignored the influence of the nitrogen present in the vitamin B concentrate and has failed to differentiate between such a purely nutritional stimulation due to the presence

of essential elements or sources of energy in a readily available form and a vitamin stimulation which would effect a continuously greater *rate* of reproduction than occurs in the controls. He did not determine rates of reproduction, but used bacterial counts to indicate rate, a procedure which has been shown not to be justified since the initial number of bacteria present, or present soon after as the result of an initial and temporarily more favorable medium, determines the numbers present at a later stage.

There can be only one possible stimulation of bacterial reproduction by vitamin B and that is a stimulation due to the vitamin B synthesized by the organism itself and such a stimulation would not be apparent in any of the experiments discussed above, since the controls themselves would be subject to the same effect.

In fact both the above organisms do elaborate vitamin B (unpublished results) and any determination of a vitamin B stimulation would be rendered rather hazardous by this fact.

CONCLUSIONS

1. The addition of vitamin B to Ashby medium exerts no stimulation of the rate of reproduction of *Azotobacter chroococcum* or *Rhizobium leguminosarum.*

2. The addition of vitamin B concentrate (Harris) to Ashby medium stimulates the rate of multiplication of *Azotobacter chroococcum* and *Rhizobium leguminosarum,* as the result of adding readily available nutrients other than vitamin B (i.e., nitrogen or source of energy). The purely nutritive effect has been confused with a vitamin effect.

3. Until further knowledge is at hand, the meaning of the term vitamin should be restricted to those substances not carbohydrates, proteins, fats or minerals essential to the growth and reproduction of suitable animals. The term cannot at present be justifiably extended to include substances necessary for the growth and reproduction of microörganisms and certainly not to indicate any substance serving to stimulate the growth or reproduction of microörganisms.

REFERENCES

BARTHEL, C. H. V., AND BENGTSSON, N. 1924 Action of stable manure in the decomposition of cellulose of tilled soil. Soil Sci., 8, 185–200.

BROADHURST, JEAN 1921 The effects of vitamine extracts in revivifying old cultures. Abs. Bact., 5, 13.

BUCHANAN, R. E. 1918 Life phases in a bacterial culture. Jour. Infect. Dis., 23, 109–125.

CHARPENTIER, C. A. G. 1921 Studien über den Einfluss des Rindvieh- und Pferdestallmistes auf die Zersetzung der Zellulose in der Ackererde. Helsingfors.[2]

FULMER, E. I., AND NELSON, V. E. 1922 Water-soluble B and bios in yeast growth. Jour. Bio-Chem., 51, 77–81.

FUNK, C., AND FRIEDMAN, L. 1922 Nutritional requirements in the growth of yeasts and bacteria. I. Vitamins. Jour. Metabol. Res., 1, 457–468.

ITANO, A. 1923 Physiological study of Azotobacter chroococcum. I. Influence of vitamin B (?) and nucleic acid on Azotobacter. Jour. Bacteriol., 8, 483–486.

LINOSSIER, G. 1919 Les vitamines et les champignons. Compt. rend. Soc. de biol., Par., 83, 346–349.

LINOSSIER, G. 1919 Les vitamines et les champignons. Compt. rend. Soc. de biol., Par., 82, 381–384.

NIKLEWSKI, B. 1912 Bodenbakteriologische Beobachtungen als Mittel zer Beurteilung von Böden. Centralbl. f. Bakteriol. (etc.), 2 Abt., 32, 209–217.

SAMMARTINO, V. 1921 Vitamins. Biochem. Ztschr., 125, 25; International Med. and Surg. Survey, 1-a, 140.

SANBORN, J. R. 1926 Physiological studies of accessory and stimulating factors in certain media. Jour. Bacteriol., 12, 1–11.

SUZUKI, V., AND SUZUKI, B. 1923 The chemical nature of vitamin D. Jour. Chem. Soc. Japan, 44, 228. Chem. Abs., 17, 2907.[2]

WAKSMAN, S. A., AND SKINNER, C. E. 1926 Microörganisms concerned in the decomposition of celluloses in the soil. Jour. Bacteriol., 12, 57–85.

WAKSMAN, S. A., AND HEUKELEKIAN, O. 1924 Microbiological analysis of soil as an index of soil fertility. VIII. Decomposition of cellulose. Soil Sci., 17, 275–291.

WERKMAN, C. H. 1925 Continuous reproduction of microörganisms in synthetic media. Science, n.s., 62, 114–115.

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[2] Original not available.

THE CORRELATION OF VARIATION IN DYE SENSITIVITY WITH GRAM CHARACTER IN CERTAIN GRAM-POSITIVE ORGANISMS[1]

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The researches reported in this paper were stimulated by the anomalous behavior met with on the part of certain Gram-positive organisms during the course of a series of investigations on the mechanism of dye bacteriostasis. The data presented were assembled to determine whether variation in Gram character is accompanied by, and thus implies, corresponding variation in isoelectric range, and also corresponding variation in resistance to gentian violet when used in nutrient media, or not.

In an investigation previously reported (Stearn and Stearn, 1926) it was found that the static action of dyes on bacteria could be explained on the assumption of the formation of an equilibrated dye-bacterial compound which behaved as one would expect an "ionic" or "salt" type of compound to behave. A basic dye was more effective as alkalinity was increased while the opposite was true for acid dyes. Moreover the equilibrated condition was demonstrated by showing the reversibility of the static action. This was demonstrated by culturing under conditions of dye concentration and pH which just inhibited growth of the organism for ninety-six hours, and then, in case of a basic dye, merely decreasing the pH, keeping all other factors constant, and remaining always within the pH range of growth for the particular organism being studied. After such a pH shift growth was obtained.

In all cases of Gram-negative organisms studied perfectly

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regular results were obtained without exception. In several cases, with Gram-positive organisms, unexpected results were obtained. These are shown in table 1 and indicated by the asterisks. This table gives results of incubation after inoculation with the indicated organism into a 0.2 per cent lactose broth, and adjustment to the pH indicated by means of phosphate buffers.

TABLE 1

	GENTIAN VIOLET DILUTION	pH 5.9	pH 6.9	pH 7.1	
Streptococcus hemolyticus (limiting pH range 5.5 to 8.0)	4,000,000	+	−	−	72-hour results
	3,000,000	−	−	−	
	2,000,000	+*	−	−	
	1,000,000	−	−	−	
		pH 6.0	pH 7.0	pH 7.6	
Staphylococcus pyogenes aureus (limiting pH range 5.6 to 8.1)	3,000,000	+	−	−	72-hour results
	2,000,000	−	−	−	
	1,000,000	+*	−	−	
		pH 5.2	pH 6.2	pH 7.2	
Bacillus cereus	4,000,000	+	+	−	24-hour results
	2,000,000	−	+*	−	
	1,000,000	−	−	−	
		pH 6.0	pH 6.6	pH 7.0	
Bacillus subtilis (limiting pH range 4.5 to 8.5)	4,000,000	−	−	−	48-hour results
	3,000,000	−	−	−	
	2,000,000	−	+*	−	

The media contained varying concentrations of gentian violet as shown.

It will be noted that in the cases indicated by the asterisk growth was obtained in an environment in which, from the general behavior shown by the table, one would not expect it. Examination of the cultures from each of the tubes exhibiting the unexpected behavior revealed the presence of both Gram-negative and Gram-positive organisms, in place of the purely positive strains with which the work had been started. Smears made

from subcultures taken from these tubes gave the same mixed picture.

The present communication embodies the report of a study of these variants. The scope of the study may be briefly outlined as follows:

1. Attempts to obtain pure Gram-negative strains (a) by mechanical selection, (b) by continued subculturing in acid and alkaline media.
2. Investigation of the persistence of the change in Gram character.
3. Investigation of the accompanying change in dye sensitivity.
4. Investigation of the accompanying change in isoelectric range.

EXPERIMENTAL

Extensive work was confined to those cultures of *Staphylococcus pyogenes-aureus*, *Bacillus cereus* and *Bacillus subtilis* obtained from the tubes whose cultures had so definitely shown a tendency to vary from the usual reaction. The *Staphylococcus pyogenes-aureus* came from the infected lip of a patient in the Pasadena Hospital. The cultures of *Bacillus cereus* and *Bacillus subtilis* came from the Natural History Museum, Washington, D. C.

A strain of *Streptococcus hemolyticus* was isolated in pure culture from the spinal fluid of a meningitis patient in the Pasadena Hospital. The work with the variant from this strain showed a behavior paralleling that of the other strains as far as it was carried.

1. Attempts to produce a pure negative strain

The general procedure consisted in streaking the culture from acid and alkaline broth, the pH of which was adjusted by means of phosphate buffers, to plain agar. After twenty-four hours incubation, colonies were examined by means of fixed smears, and stained by the classic Gram technique. The colonies showing the greatest preponderance of Gram-negative organisms were transferred to 0.2 per cent lactose broth adjusted to a pH of 5.2, and those showing a preponderance of Gram-positive organisms were transferred to broth adjusted to a pH of 7.8 to 8.0   These

resulting cultures were again streaked to plain neutral agar and the same process of mechanical selection and subculturing into acid and alkaline media repeated.

The results obtained from the daily examination of a large number of the isolated colonies on twenty-four-hour plates, streaked from the variant of *Staphylococcus pyogenes-aureus*, are given in table 2.

TABLE 2

DAY	NUMBER OF COLONIES EXAMINED	NUMBER OF COLONIES MIXED	NUMBER OF COLONIES MOSTLY BLUE	NUMBER OF COLONIES MOSTLY RED
1	26	19	3	4
2	20	14	6	—
3	25	22	3	—
4	26	24	2	—
5	26	24	2	—
6	25	23	—	2
7	22	16	3	3
8	39	28	10	1
9	20	15	5	—
10	43	29	6	8
11	54	34	20	—
12	77	70	4	3
13	67	61	1	5
14	72	58	3	11
15	73	61	4	8
16	75	71	1	3
17	15	15	—	—
Totals........	705	584	73	48

The part of the colony taken for making the smear did not seem to affect the relative number of red and blue cocci.

In general, pure mechanical selection did not seem to increase significantly the preponderance of Gram-negative organisms.

Incubating in media of a comparatively low pH for long periods of time did, however, in case of all three organisms give a product which, though still mixed, was not more than 50 per cent Gram-positive. No cases of pure negative smears were noted.

The change in staining character, as a statistical change, is extremely slow. All three of the organisms studied gave smears

of the original variant which were preponderantly Gram-positive. It was only after months of subculturing at a low pH that a strain statistically equal in positive and negative and individuals could be obtained.   The change is a reversible one, but it is significant to note that reversion is as slow and as difficult of accomplishment as the original change.   The strain of *Bacillus cereus*, after it had been cultured for months at a low pH and was yielding smears with about the same number of negative as of positive organisms, was then cultured in broth at a higher pH.   Even after three months of such culturing smears still showed Gram-negative organisms, though finally to an extent much less than when the culturing at the higher pH was begun.

It should be stated that on the same slide with the smears of variant under examination there was also placed a smear of the original purely Gram-positive organism which had been maintained at a high pH.   This insured identical treatment of the two smears in the staining technique and permitted comparison of the variant with the orthodox strain.   This latter retained its pure Gram-positive character throughout the course of the work.

The slowness of the reversion of the variant, as well as the fact of mixed strains, seems to the authors to constitute fairly definite proof that the Gram reaction of an organism does not depend exclusively, or even primarily, on the surface condition of the organism, but is due to characteristics more fundamental.   These have been fully discussed previously (Stearn and Stearn, 1924 a and b).

To meet possible objections that mixed smears might be due to mixed or impure cultures, a large number of single cell cultures were obtained, using a modification of Orskov's method (1922). These cultures, in all cases showed the same mixed character as did the cultures from which they were obtained.   It should also be remarked that a control culture of the Gram-negative organism, *Bacillus coli*, was "carried along" through similar procedures for long periods of time, but in no case was any change in Gram character noted.   In no cases was a mixed smear obtained even after long culturing at a high pH.

TABLE 3

Bacillus cereus

DILUTION OF GENTIAN VIOLET	VARIANT, SHOWING ABOUT 50 PER CENT GRAM-NEGATIVE RODS			ORIGINAL STRAIN SHOWING A PURE GRAM-POSITIVE SMEAR		
	pH 5.2	pH 6.2	pH 7.7	pH 5.2	pH 6.2	pH 7.7
1,000,000	+	−	−	−	−	−
	+	−	−	−	−	−
	+	+	−	−	−	−
2,000,000	+	−	−	−	−	−
	+	−	−	−	−	−
	+	+	−	−	−	−
4,000,000	+	+	−	−	−	−
	+	+	−	+	+	−
	+	+	−	+	+	−
6,000,000	+	+	−	+	+	−
	+	+	+	+	+	−
	+	+	+	+	+	+

Results are given for twenty-four, forty-eight and seventy-two hours incubation.

TABLE 4

Bacillus subtilis

DILUTION OF GENTIAN VIOLET	VARIANT, SHOWING ABOUT 50 PER CENT GRAM-NEGATIVE RODS			ORIGINAL STRAIN SHOWING A PURE GRAM-POSITIVE SMEAR		
	pH 5.2	pH 6.2	pH 7.7	pH 5.2	pH 6.2	pH 7.7
1,000,000	−	−	−	−	−	−
	−	−	−	−	−	−
	−	−	−	−	−	−
2,000,000	+	+	−	−	−	−
	+	+	−	−	−	−
	+	+	−	−	−	−
4,000,000	+	+	+	−	−	−
	+	+	+	+	+	−
	+	+	+	+	+	−
6,000,000	+	+	+	+	+	−
	+	+	+	+	+	+
	+	+	+	+	+	+

Results are given for twenty-four, forty-eight and seventy-two hours incubation.

2. Variation in sensitivity to dyes

Two distinct series of experiments were carried out. The first was by means of direct transfer from colonies on agar showing a tendency toward Gram-negativeness, and the other was by the transfer of two drops of a twenty-four-hour acid and alkaline broth culture, each into sterile distilled water. These tubes were thoroughly shaken and allowed to stand for ten minutes after which one drop was inoculated into 0.5 per cent lactose gentian violet broth. The variant which had been cultured at low pH and that which had been cultured at high pH were always run parallel, using identical media.

TABLE 5

Staphylococcus pyogenes-aureus

DILUTION OF GENTIAN VIOLET	VARIANT, SHOWING ABOUT 50 PER CENT GRAM NEGATIVE RODS			ORIGINAL STRAIN SHOWING A PURE GRAM-POSITIVE SMEAR		
	pH 6.0	pH 7.0	pH 7.7	pH 6.0	pH 7.0	pH 7.7
2,000,000	+	−	−	−	−	−
3,000,000	+	−	−	+	−	−
4,000,000	+	+	+	+	+	−

Results are given for seventy-two hours incubation.

Tables 3 and 4 give the results of one typical experiment on two of the three organisms studied. In both cases the variant came from a single cell culture. Comparison is made with the non-variant strain kept continuously at a high pH. These tables show the decrease in sensitivity to dye paralleling the partial loss of Gram-positiveness. This difference in sensitivity to gentian violet on the part of the two strains of the same organism was repeatedly confirmed.

In the case of the results given in table 5 for *Staphylococcus pyogenes-aureus*, the sensitivity of the variant is compared with that found for the original strain at the beginning of the work. Results are given for seventy-two hours incubation.
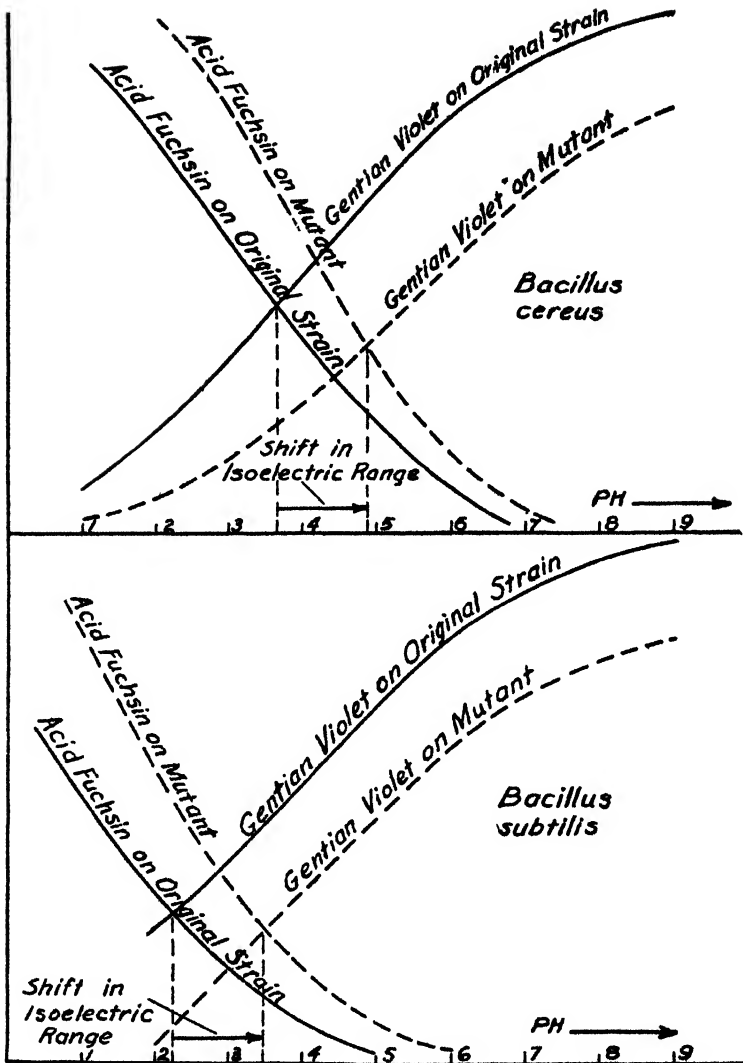
FIG. 1. SHOWING THE SHIFT IN ISOELECTRIC RANGE PARALLELING THE CHANGE IN
GRAM CHARACTER AND IN SENSITIVITY TO GENTIAN VIOLET

The broken line gives the behavior of the variant, the continuous line that of
the original strain.   Abscissae give pH values while ordinates represent intensity
of retained color, after decolorizing with acetone, on an arbitrary comparative
scale.   For the experimental method of determining these curves see Stearn and
Stearn, 1924a.

3. Changes in isoelectric range

The character most important in determining whether or not gentian violet is strongly retained by an organism has been shown by Stearn and Stearn (1925) to be the specific acidity of the organism. By this is meant its tendency to combine with basic substances such as gentian violet. The organisms most strongly Gram positive are most acidic in nature. The amphoteric nature of bacteria has also been demonstrated by the authors (Stearn and Stearn, 1923), and it has been shown that the isoelectric range is a measure of the acidic strength of bacteria. The more strongly acidic an organism is, the lower is the pH of the isoelectric range. One might expect, then, with loss of Gram positiveness, a corresponding shift of isoelectric range to a higher pH.

The isoelectric ranges of *Bacillus cereus* and *Bacillus subtilis* were determined for the original strains and for the variants. The results are given graphically in figure 1. In both cases a distinct shift is noted in the isoelectric range toward a higher pH.

DISCUSSION

One of the outstanding questions arising from the experimental work presented in this paper is whether the varation of strains, discovered in the cases of the Gram-positive organisms growing under adverse environmental conditions, is due to the purely chance presence of an already largely adapted variant which will develop more rapidly and prolifically than its less fortunate brothers and thus tend to breed a more resistant strain by selection, or whether, as one approaches a limit in imposing adverse environmental conditions on a growing organism, one reaches a fairly definite point on the one side of which no growth takes place but on the other side of which there is a gradual tendency toward adaptation on the part of the majority of the organisms present. Such a question is not easy to answer and will probably form the basis for considerable future experimentation.

The study of variation by watching the change in Gram character, though interesting and important in itself, will probably appear at first sight as a comparatively trivial matter and one

of limited importance. Such, however, will be by no means the case when it is shown that the Gram reaction need not be confined to an arbitrary classification of organisms, but that, in the case of any individual species, variation in Gram character is paralleled by, and is thus a reflection of, variation in sensitivity to dyes. That Gram-positive organisms are, on the whole, more sensitive to basic dyes than Gram-negative ones is generally known. The experimental evidence presented in this paper goes farther, however, and shows that, in the case of the *same* species of organism, loss in Gram-positiveness is accompanied by decreased sensitiveness to the basic dye gentian violet.

Regardless of the reason for the first appearance of the variant strains, two facts are of note as brought out in this study.

1. After its original appearance, the variant persists even after long subculturing under conditions favorable to the "orthodox" strain.

2. It has been found impossible to obtain a culture in which all of the organisms are individually variant, though attempts to obtain such a culture were made both by mechanical selection and by long continued subculturing under adverse environmental conditions such as at a low pH.

Reference to table 2 shows that, out of over 700 colonies examined, about 83 per cent were mixed colonies in which neither Gram-positive nor Gram-negative organisms predominated to any considerable degree, about 10 per cent were mostly Gram-positive while about 7 per cent gave almost pure smears of the Gram-negative variant.

Regarding the first of the two facts given above, it should be stated, to avoid misconception, that the statistical persistence of the variant strain is definitely dependent on its environmental conditions. Under proper conditions, there is a tendency to an extremely slow reversion to the original strain, i.e., when culturing conditions favorable for the development of the original strain are used. In this work, however, though such a tendency was clearly indicated, complete reversion was never obtained.

Another significant fact brought out in this study is that the formation of these chance variants has occurred, in our experi-

ments, only in the case of Gram-positive organisms. No cases of a chance alteration of a Gram-negative to a Gram-positive organism have been noted. Such a change is fundamentally different, however, from the change—Gram-positive to Gram-negative. In the latter case there is the loss of a character—the retaining power for gentian violet. It is more or less absurd to speak of the loss of negativeness. The change, negative to positive, entails not the loss but the acquisition of a character—the retaining power for gentian violet—and one might well expect chance loss of positiveness to appear much more frequently in the case of a typical Gram-positive organism than the acquisition of positiveness by a typical negative organism. The latter change may possibly be induced, but it would be a matter of forcing the character onto the organism and the methods would be, in general, more rigorous.

Churchman (1921), though he was unable to train *Bacillus subtilis* to grow in the presence of gentian violet, did succeed in obtaining a strain of *Bacterium coli* whose sensitiveness to gentian violet was markedly increased over that of ordinary cultures. This was accomplished, not by special treatment of the original culture, but by a technique of selection in which increasingly dilute suspensions were streaked on divided agar plates until finally growth occurred only on the side of the plate free from gentian violet. The results are explained by Churchman as due to the fact that only a comparatively small proportion of the organisms in any strain of *Bacterium coli* will grow in the presence of the dye, but that with heavy inoculations there are enough of those which will grow to produce satisfactory growth. The "gentian violet positive" strain thus developed was still Gram-negative in reaction, and thus the conclusion was reached that "the factor which determines the reaction of an organism to the Gram process is not the same as the factor which determines its growth in the presence of gentian violet." No data as to concentration of dye used nor pH of media are given in the report, so that critical interpretation of the results is impossible.

In the attempts to acclimatize *Bacillus subtilis* to gentian violet it was reported that in some of the tubes in which there was a

concentration of dye 1:1,000,000 there were a "few organisms" developed. No report of the Gram character of these "individual cases" is given, though it is possible that had they been examined, it would have been found that they contained both the Gram-positive and Gram-negative organisms in the same way that those seemingly chance "individual cases" reported in this study did, and which in the present case furnished the starting point for the work on the Gram-positive organisms.

If Churchman's explanation of the development of a gentian violet positive strain of *Bacterium coli* is valid, his results furnish some basis for the idea that, in the appearance of these variant strains, we are dealing with variants already present in any culture, and which require only an environment which, though adverse to the culture as a whole, still permits them to multiply so that we have a partially selective growth.

The results, however, certainly do not warrant any definite conclusion that the factor which determines Gram character is not the same as that which determines sensitiveness to gentian violet. The comparatively large amount of systematic work done by the present authors in their various studies in which determining factors were discovered and definitely controlled indicates a close parallel between Gram character and sensitiveness to basic dye. The apparent contradiction to this idea in the work of Churchman reviewed here is one of degree only. Gram-positive organisms may lose the retaining power for gentian violet, and, though their sensitiveness to this dye is noticably decreased, paralleling this loss, they are even yet more sensitive than ordinary typical Gram-negative strains. The Gram technique is a rather crude and rigorous procedure from the point of view of a live organism, and one might well expect slight increases in retaining power for gentian violet to be sufficient seriously to affect the viability of a growing culture, even though they were so small that, with such vigorous decolorizing as given in the gram technique, no difference could be noted—using such crude methods as visual observation and even clouding this observation with a strong counterstain. Gram negative organisms which counterstain to a brilliant red with the proper dye might, and often would,

appear far from completely decolorized if no counterstain were used; the phenomenon is one of gradation in effect.

From this more general point of view it may well be expected that an organism may become distinctly more sensitive to gentian violet but still be classified as Gram-negative, while another organism may lose its Gram-positiveness but still retain a fairly high sensitiveness to gentian violet.

It should finally be pointed out that the experimental changes brought about in the variants as described in the foregoing paper were *not* brought about by acclimatization methods. The decreased sensitivity to gentian violet on the part of the variants was not due to the acclimatizing effect of culturing for long periods of time in the presence of small amounts of the dye. The shift in isoelectric range, the change in Gram character and the decreased sensitivity to gentian violet are all reflections of some more fundamental change which the organisms have undergone.

SUMMARY

1. Chance variants were discovered in several cases of strongly Gram-positive organisms growing under adverse environmental conditions.

2. This variation has been shown to persist.

3. It has not yet been found possible, either by mechanical selection, or by the modification of growth media either by exaggeration or modification of the adverse conditions, to isolate a pure strain of the variant.

4. Experiments have been adduced to show that, though the original variant seemed to be due to chance, any subsequent change in the strain is very gradual. By long subculturing under a certain constant set of conditions the variant tendency may be greatly increased, while under another constant set of conditions a tendency to an extremely slow reversion to the original strain is noted.

5. Loss of Gram-positiveness, i.e., decrease in affinity for basic dye, has been shown to parallel exactly the decrease in sensitiveness to basic dye.

6. Loss of Gram-positiveness has been shown to be accom-

panied by a definite shift in the isoelectric range of the organism. This shift is in the direction which one would expect from the study of the isoelectric ranges of the typical Gram-positive and Gram-negative organisms.

7. All of the results obtained in this study are completely in accord with, and furnish confirmation of, the general theory of the Gram reaction and of dye bacteriostasis as developed during recent years by the author.

In conclusion the author wishes to acknowledge her indebtedness to Dr. M. P. Ravenel of the Department of Preventive Medicine of the University of Missouri, for the interest he has shown during the course of this work, and for assistance in the preparation of this manuscript.

REFERENCES

CHURCHMAN 1921 Jour. Exper. Med., **33**, 569.
ORSKOV 1922 Jour. Bacteriol., **7**, 537.
STEARN AND STEARN 1923 Jour. Bacteriol., **8**, 567.
STEARN AND STEARN 1924a Jour. Bacteriol., **9**, 463.
STEARN AND STEARN 1924b Jour. Bacteriol., **9**, 479.
STEARN AND STEARN 1925 Jour. Bacteriol., **10**, 13.
STEARN AND STEARN 1926 Jour. Bacteriol., **11**, 345.

THE EFFECT OF GELATIN ON THE BACTERIAL CONTENT OF ICE CREAM MIX[1]

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INTRODUCTION

During the past few years the bacterial content of ice cream has been given considerable attention by sanitary specialists, both from the qualitative and quantitative standpoints. Among the possible factors contributing to the bacterial content of ice cream is gelatin. The use of gelatin in ice cream at first met with strong opposition, due to a popular prejudice based on a misunderstanding of the sanitary quality of this animal product. Although this prejudice has been largely overcome, and gelatin is now almost universally used in the manufacture of ice cream, the idea still prevails in the minds of some that gelatin is a prolific source of contamination in the ice cream mix, and should be used as sparingly as the stability of the product will permit.

In view of the small percentage of gelatin used in ice cream, it seemed illogical that it should be of serious consequence from the standpoint of bacterial numbers. Since most ice cream manufacturers use only 0.5 per cent, or even less of gelatin in the mix, it would necessitate a very large number of organisms in the gelatin materially to increase the bacterial count of the mix. A brief review of the literature on the bacterial content of gelatin indicates the correctness of this assumption.

REVIEW OF LITERATURE

In 1912, Gordon reported the bacterial content of 20 samples of gelatin, varying between 200 and 30,000,000 per cubic centi-

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meter. Later, Bahlman (1914) examined 14 samples of gelatin for total numbers of bacteria and found them to contain from 300 to 6,800,000 per cubic centimeter. Ayers (1914) predicted that a study of the bacteriology of gelatin would lead to a method of detecting gelatin of poor quality. Gordon (1915) recommended the addition of gelatin to ice cream mix, only after it has been dissolved in boiling water. He stated that gelatin added in this manner should not contain more than 1000 to 2000 bacteria per cubic centimeter. Hammer (1917) reported the analysis of five samples of gelatin, varying in bacterial content from 35 to 113,000,000 per gram. Ellenberger (1919) found that by dissolving gelatin in boiling water, the average bacterial content of 15 samples was reduced from approximately 390,000 to 530 per gram.

In 1920 the New York City Board of Health specified that gelatin which contained any bacteria of the colon group should be considered adulterated and could not be sold in the city. Hall and Houtz (1923) made a study of twelve different tests for quality in gelatin and concluded that only the bacteria count and the jelly strength test were necessary to determine the value of gelatin. Parfitt (1923a) found the total counts on 15 samples to range between 100 and 60,000,000 bacteria per gram, and the colon counts between "less than 100" and 35,000,000 per gram.

In discussing the various signs of low quality gelatin, Burke (1923) stated that bad gelatin, among other factors, was high in bacterial count. He submitted analyses ranging from 200,000 to 9,000,000 bacteria per gram and stated that gelatin might add from 5000 to 500,000 bacteria per cubic centimeter to the mix. Parfitt (1923b) defined a good gelatin as one having, among other qualities, a nearly neutral reaction, a low bacterial count (not to exceed 5000) and no *B. coli* types.

The Oklahoma Experiment Station (1923–1924) conducted a study of approximately 200 samples of gelatin. The results of their work indicated that the high grades of gelatin were less soluble in water, had practically no odor, and a low bacterial content. Tracy in 1924, and also Brannon and Tracy (1925) reported a bacteriological study of 142 samples of gelatin. They found no relation between the cost of the gelatin and the quality

as evidenced by the bacterial count. Of the samples studied, 8 per cent were sterile, 50 per cent contained less than 20,000 bacteria per gram, and 31 per cent contained in excess of 1,000,000 per gram. They concluded that the addition of gelatin to ice cream resulted in a very slight increase in the number of bacteria in the mix. Turnbow and Milner (1926) found that when gelatin was given the ordinary liquefying test, the high hydrogen-ion concentration of many gelatins prevented liquefying bacteria which were present from growing. By changing the pH of many gelatins, the presence of large numbers of gelatin liquefying bacteria was revealed. Combs (1926) studied the relative value of various tests on gelatin and concluded that the bacterial examination, the swelling property, the jelly strength, and the solubility were all important tests, and should be considered in the selection of gelatin. Analyses of six samples varied between 300 and 13,500 bacteria per gram with an average of 5160.

Ambrose (1926) was of the opinion that a discussion of the different grades of gelatin should include a consideration of the bacterial content. His work indicates that the bacterial flora of gelatin is not, as a rule, made up of a very high per cent of heat resistant bacteria. One sample containing 5000 bacteria per gram was reduced to 800 by heating to 180°F. for five minutes; another was reduced from 6,100,000 to 250 per gram by heating to 140°F. for five minutes. Sommer (1927) regarded the "keeping test" of gelatin as of little value since it is more dependent on the acidity than on the actual bacterial content. This conclusion is substantiated by the conclusion of Turnbow and Milner previously referred to.

COLLECTION OF SAMPLES

The work reported in this paper constitutes a summary of 146 analyses of 50 samples of gelatin. In connection with an investigation of gelatin to be used in ice cream, a large number of samples were available for bacterial analysis. Thirty-one different grades of gelatin, representing the product of eight gelatin manufacturers were included in the samples analyzed. These were

obtained directly from the various companies. The remaining samples were collected from barrels of gelatin in use in ice cream plants.

Sampling

A 10-gram sample of dry gelatin was weighed on sterile paper and placed in a dilution blank, from which appropriate dilutions were made. In a few instances a 1-gram sample was accurately weighed on analytical balances for the sake of comparison with results obtained with 10-gram samples weighed on less sensitive torsion balances. The counts, represented the number of bacteria per gram of gelatin. The dilution blanks were heated to 45 degrees in order to facilitate solution of the gelatin.

Plating

The procedure recommended in the Standard Methods for Bacteriological Examination of Milk was followed in making all the determinations. The medium used was plain, beef extract agar with Difco peptone, prepared according to Standard Methods. The reaction was adjusted to pH 7.0

RESULTS

In table 1 are recorded the bacterial counts obtained on fifty samples of gelatin.

An examination of table 1 will show that the bacterial counts in these fifty samples of gelatin varied from 10 to 108,000,000 per gram, with an average of 12,738,800. The bacterial counts recorded in table 1 may be summarized as follows:

		Bacteria per gram
14 per cent of the samples contained	less than	1,000
22 per cent of the samples contained	less than	10,000
34 per cent of the samples contained	less than	100,000
48 per cent of the samples contained	less than	1,000,000
32 per cent of the samples contained	more than	10,000,000
16 per cent of the samples contained	more than	35,000,000

Table 1 also shows the number of separate analyses made of each sample of gelatin.   The counts reported are the average of all the

TABLE 1

The bacterial content of 50 samples of gelatin and the calculated number of bacteria added to each gram of ice cream mix when 0.5 per cent of gelatin is used

SAMPLE NUMBER	NUMBER OF ANALYSES	BACTERIA PER GRAM	INCREASE IN BACTERIAL COUNT OF RAW MIX WHEN 0.5 PER CENT OF GELATIN IS ADDED	SAMPLE NUMBER	NUMBER OF ANALYSES	BACTERIA PER GRAM	INCREASE IN BACTERIAL COUNT OF RAW MIX WHEN 0.5 PER CENT OF GELATIN IS ADDED
1	2	10	0.05	29	18	3,970,000	18,850
2	1	100	0.5	30	3	5,260,000	26,300
3	3	170	0.85	31	2	6,850,000	34,250
4	2	380	1.9	32	2	7,150,000	37,750
5	2	450	2.2	33	3	8,560,000	42,800
6	2	650	3.2	34	1	9,700,000	48,500
7	2	750	3.7	35	2	10,325,000	51,625
8	2	1,450	7.2	36	16	14,950,000	74,750
9	2	3,500	17	37	1	16,100,000	80,500
10	3	4,500	22	38	4	19,500,000	87,500
11	3	10,000	50	39	4	20,100,000	100,500
12	1	11,000	55	40	4	20,400,000	102,000
13	2	12,000	60	41	2	22,100,000	110,500
14	2	16,000	80	42	2	24,922,000	124,610
15	3	18,000	90	43	2	36,300,000	181,500
16	2	68,000	340	44	4	37,750,000	188,750
17	3	74,000	370	45	4	38,000,000	190,000
18	3	248,000	1,240	46	1	43,000,000	215,000
19	2	380,000	1,900	47	4	48,500,000	242,500
20	2	387,000	1,935	48	1	51,000,000	255,000
21	1	400,000	2,000	49	1	71,200,000	356,000
22	1	515,000	2,575	50	2	108,000,000	540,000
23	3	618,000	3,090				
24	3	842,000	4,210	Total......	146		
25	3	1,527,000	7,635				
26	4	1,745,000	8,725	Average...		12,738,800	63,694
27	2	2,720,000	13,600				
28	2	3,700,000	18,525	Median....		1,636,000	8,180

analyses on the sample involved.   In this table is also given the calculated number of organisms that each of these various samples of gelatin would add to each gram of ice cream mix if used in

0.5 per cent quantities. For example, sample no. 50, containing 108,000,000 bacteria per gram of gelatin, would increase the bacterial count of the raw ice cream mix 540,000 per gram if used in 0.5 per cent amounts.

The results shown in table 1 indicate that only in extreme cases would gelatin be an important source of contamination from the standpoint of total numbers of bacteria added to unpasteurized ice cream mix. On the other hand, the use of any one of the first 26 samples reported in table 1 would increase the count of the ice cream mix less than 10,000 per gram when used in 0.5 per cent quantities.

A study of the records of bacterial counts on raw ice cream mix from analyses made at this station, covering a period of several years, shows that the counts ranged from 120,000 to 93,000,000 per gram, with an average or 12,000,000 bacteria per gram. About half of the mixes analyzed contained in excess of 7,000,000 bacteria per gram. Assuming 7,000,000 to be a representative count for raw ice cream mix, and the experimental error of the plate count to be 10 per cent, any gelatin containing less than 140,000,000 bacteria per gram could be added to the mix in 0.5 per cent amounts and the resulting increase in bacterial count would be within the limits of the assumed error of the method. This would include all of the gelatins reported in this paper, and also all of the samples examined by Brannon and Tracy (1925).

On the other hand, if the bacterial content of the raw mix is low, i.e., 500,000 per gram, gelatin might be a source of contamination worthy of consideration. The bacterial count of a raw ice cream mix originally containing 500,000 bacteria per gram would be appreciably affected by any gelatin that added more than 50,000 bacteria to each gram of mix. Reference to table 1 shows that by using 0.5 per cent of any of the last 16 samples of gelatin, the count of such a mix would probably be sufficiently affected to be detected by the plate method. On the same hypothesis, sample number 50 could be used in any mix containing more than 5,400,000 per cubic centimeter without noticeably affecting the results of the plate count. If this assumption is correct, it could be used in over 85 per cent of the raw mixes studied at this station.

It appears from this discussion that the bacterial content of the average raw ice cream mix is so high that the contamination from gelatin would be rendered insignificant, except when very poor gelatin is used or when the bacterial content of the raw mix is unusually low.

TABLE 2

Effect of pasteurizing gelatin at 145°F. for thirty minutes

BACTERIAL COUNT PER GRAM BEFORE PASTEURIZATION	BACTERIAL COUNT PER GRAM AFTER PASTEURIZATION	PER CENT EFFICIENCY OF PASTEURIZATION
40	15	52.5
70	5	92.9
120	120	0.0
130	60	53.9
240	220	8.4
270	210	22.3
380	270	29.0
1,310	1,300	0.8
2,890	1,160	59.9
2,950	75	97.5
4,600	350	92.4
5,450	120	97.8
34,000	2,300	93.3
48,000	150	99.7
72,500	4,900	93.3
147,000	62,000	57.9
278,000	1,270	99.6
293,000	4,900	98.4
365,000	330	99.9
385,000	40	99.9
408,000	2,200	99.5
5,268,000	1,200	99.9
Average 332,588	3,781	98.87

Effect of pasteurization

The question immediately arises as to whether an increase in the bacterial count of a raw mix, resulting from the use of poor gelatin would be of any consequence in a pasteurized mix.   Obviously, this depends on the per cent of the bacteria in gelatin which will resist the pasteurizing temperature.

In practice some ice cream makers dissolve the gelatin in hot

water and add it after the mix has been pasteurized; others add the gelatin directly to the mix before pasteurization. Ellenberger (1919) showed that by dissolving gelatin in hot water (160° to 170°F.) the average count of 15 samples of gelatin was reduced from 388,162 to 1172 bacteria per gram or a reduction of 99.7 per cent.

In table 2 are shown the bacterial counts on 22 samples of gelatin before and after pasteurization at 145°F. for thirty minutes. (The 22 samples included in this experiment are not represented in table 1.) It will be noted that the efficiency of pasteurization varies from 0 to 99.9 per cent, and that for the entire 22 samples, 98.87 per cent of the bacteria were destroyed. Attention is also called to the fact that, with one exception, in all of the samples containing more than 3000 bacteria per gram, more than 90 per cent of the organisms were killed by pasteurization. Those samples which exhibited a bacterial flora very resistant to heat had relatively few bacteria to begin with, and therefore, would not offer a serious source of contamination to ice cream even if they all survived. The largest number of bacteria to survive pasteurization in any of the 22 samples was 62,000 per gram.

The results in table 2 indicate that if gelatin is pasteurized with the other ingredients of the mix, a sufficiently large per cent of the bacteria will be killed to render it a negligible source of contamination. If the average per cent efficiency of pasteurization obtained with these 22 samples (98.87) were obtained with a sample containing 108,000,000 per gram (sample No. 50) there would remain only, 1,220,000 bacteria per gram; this would increase the count of each gram of ice cream mix, 6100 if 0.5 per cent were used. It is probable that some gelatins containing large numbers of organisms might have a bacterial flora of heat resistant types. Such a gelatin would be an important source of contamination even if pasteurized. No such gelatin, however was encountered in the 22 samples herein reported.

Bacterial flora of gelatin

Table 3 shows the results of differential counts of eight samples of gelatin. The total count, the per cent of acid forming

types, acid and gas forming types, liquefying types, and neutral types were determined.   The differentiation of the types of bacteria was made on a basis of their action on lactose and on gelatin. The acid forming types, and the acid and gas forming types were determined by serial dilution in Durham lactose fermentation tubes, containing brom-thymol-blue as an indicator.   The highest dilution showing acid was recorded as the number of acid formers, and the highest dilution showing gas was recorded as the number of acid and gas formers.   The liquefying types were determined by serial dilution in tubes of nutrient gelatin.   The highest dilution which failed to solidify in ice water, after incuba-

TABLE 3

The per cent of various types of organisms found in 8 samples of gelatin

SAMPLE NUMBER	TOTAL COUNT—TOTAL NUMBER OF BACTERIA BY PLATE METHOD	PER CENT OF			
		Acid forming types	Acid and gas types	Gelatin liquefying types	Neutral types
		per cent	per cent	per cent	per cent
1	515,000	19.0	2.0	0.0019	79.0
2	3,445,000	7.0	1.0	0	92.0
3	2,800,000	9.0	4.0	0	87.0
4	2,500,000	0.02	0.02	0.04	99.0
5	1,000,000	10.0	10.0	0	80.0
6	380,000	1.0	0.3	0.02	98.0
7	586,000	9.0	2.0	0.0	89.0
8	890,000	1.0	1.0	0.0	98.0

tion for forty-eight hours at 37°C., was recorded as the number of liquefying types.   The neutral types were determined by subtracting from the total count the sum of the other types.   There is, obviously, a large per cent of error in any serial dilution method, but it may serve to throw some light on the relative per cent of various types of bacteria present.

It will be noted in table 3 that there was a very small per cent of liquefying types of bacteria present in the eight samples analyzed; five of them contained no liquefying types.   The acid formers ranged from less than 1 to 19 per cent, and the acid and gas formers varied between 0.02 and 3.0 per cent of the bacterial

flora. The majority of the bacteria in these samples of gelatin had no action on lactose or gelatin and were classed as neutral types.

Variation in bacterial counts of gelatin

Considerable variation was noted in results when several separate analyses were made of the same sample of gelatin. Even though great care was used to make duplicate determinations as nearly identical as possible, widely varying counts were frequently obtained. The same discrepancies are noted in making quantitative determinations of soil, manure, etc. Such variations are probably due, in part, to the inclusion in certain weighings, of masses of material (soil, manure, or gelatin) containing excessive numbers of organisms.

In order to show the degree of variation to be expected in bacterial counts on gelatin, 100 plates were poured from a single sample of gelatin. Five 10-gram samples were weighed on torsion balances and placed in warm 90 cc. sterile dilution blanks. From each of these, dilutions of 1:1000 were made and ten plates were poured from each, making a total of 50 plates. In addition, five 1-gram samples were weighed accurately on analytical balances and placed in warm 99 cc. sterile dilution blanks. From each of these, 1:1000 dilutions were prepared and ten plates poured from each as before. There were, therefore, ten series of ten plates each, 50 of which were from 10-gram samples weighed on ordinary balances, and 50 from 1-gram samples, more accurately weighed on analytical balances.

In table 4 the degree of variation is expressed in terms of the probable error of single and duplicate plates. It will be seen that the average of 100 plates is 357,690 bacteria per gram. For convenience of interpretation this will be arbitrarily assumed to represent the "true count," since it was obtained by averaging more plates than would ordinarily be made on a single sample. The probable error of a single plate in this series of variates is ±38,561 or 10.7 per cent of the "true count." This means that there is a fifty-fifty chance that any single plate made on this sample of gelatin would yield results between the limits 357,690

±38,651 (319,129–396,251). In other words, there is a fifty-fifty chance that a single plate would give results within 10.7 per cent of the count which would have been obtained if 100 plates had been made instead of only one. If the average of duplicate plates is taken, then the probable error is shown to be ±27,267 or ±6.2 per cent. There is an even chance that the average of duplicates would not deviate more than 6.2 per cent from the "true count."

TABLE 4

The variation in 100 plates on a single sample of gelatin

	MEAN COUNT PER GRAM	PROBABLE ERROR OF SINGLE PLATE, IN BACTERIA PER GRAM	PROBABLE ERROR OF SINGLE PLATE, IN PER CENT	PROBABLE ERROR OF DUPLICATE PLATES, IN BACTERIA PER GRAM	PROBABLE ERROR OF DUPLICATE PLATES, IN PER CENT	3.2 × PROBABLE ERROR OF DUPLICATE PLATES, IN BACTERIA PER GRAM	3.2 × PROBABLE ERROR OF DUPLICATE PLATES, IN PER CENT
First 50 plates, 10-gram samples weighed on torsion balances..........	386,020	±25,321	±6.5	±17,904	±4.6	±57,297	±14.8
Second 50 plates, 1-gram samples weighed accurately on chemical balances...............	329,360	±40,025	±12.1	±28,302	±8.5	±90,566	±27.4
Entire 100 plates combined.................	357,690	±38,561	±10.7	±27,267	±6.2	±87,254	±24.3

Obviously, it is desirable to establish limits within which more than 50 per cent of the samples will fall. This may be done by multiplying the probable error by a factor, thereby widening the limits to include more than 50 per cent of the variates. Biometricians frequently use the factor 3.2 which includes approximately 97 per cent of the variates. In other words, it establishes limits within which there is a 30 to 1 chance that the results of a determination will fall. In table 4 it will be noted that the probable error of duplicate plates multiplied by 3.2 gives the value ±87,254 or 24.3 per cent. That is to say, there is a fair degree of certainty (30 to 1 chance) that the average of duplicate

plates made from this gelatin will not fall outside the limits of 357,690 ±87,254 (270,436–444,944). It is fairly safe, therefore, to assume that an average of duplicate plates on this gelatin will not be more than 24.3 per cent from the mean. However, it is not to be implied that the value 24.3 per cent may be applied to all bacterial counts on gelatin. This figure is strictly applicable only to this particular sample and the particular conditions under which the determinations were made. Although these figures cannot be applied too strictly, they may throw some light on the expected variation of a bacterial analysis of gelatin.

It is of interest to note that the fifty plates made from 1-gram samples, weighed accurately on analytical balances, showed almost twice as large a probable error as the plates from 10-gram samples weighed on torsion balances. The first series of 50 plates showed a probable error for single plates of ±6.5 per cent, whereas the second series, weighed more accurately in 1-gram samples, has a probable error of ±12.1 per cent. This apparent inconsistency is believed to be as stated earlier, due, in part at least, to the inclusion in some of the weighings and not in others of particles containing excessive numbers of bacteria. Such discrepancies in a 1-gram sample would probably cause much greater variation than in a 10-gram sample, even though weighed on less accurate balances. Insufficient work has been done to state whether this condition would always obtain, but it is believed that the results of this single experiment illustrate the necessity of using larger samples of gelatin in bacterial analysis.

SUMMARY

Bacterial counts are reported on 50 samples of gelatin. Thirty-one different grades of gelatin representing the products of eight gelatin manufacturers were analyzed. The remainder of the samples were obtained from ice cream plants and may have been duplicates of some of the grades obtained direct from the gelatin factories.

The number of bacteria in these samples of gelatin is shown to vary from ten per gram to 108,000,000 per gram. From these data, calculations have been made, showing the number of organ-

isms which each sample of gelatin would add to a gram of ice cream mix if used in 0.5 per cent amounts.   The increase in the bacterial count of ice cream mix which would result from the use of these gelatins is shown to vary from less than one organism per gram to 540,000 per gram.   Gelatin, therefore, in extreme cases may be an important source of contamination of ice cream, but the use of the gelatin containing the highest count in this group of samples on a hypothetical basis, would not appreciably increase the plate count of 85 per cent of the raw mixes analyzed at this station during the past six years.   Gelatin added to the mix after pasteurization, or poor quality gelatin added to a mix already low in bacterial count may result in a noticeable increase in the plate count.

Twenty-two samples of gelatin were pasteurized at 145°F. for thirty minutes.   The average reduction in count was 98.87 per cent.   With one exception, a reduction greater than 90 per cent was obtained with all samples containing more than 3000 bacteria per gram.   If the 22 samples pasteurized in this experiment are representative, gelatin is not likely to be a serious source of contamination when pasteurized with the other ingredients of ice cream mix.

The results of qualitative analyses of eight samples of gelatin show that the gelatin liquefiers varied from none to 0.04 per cent, and that the lactose-fermenting organisms constituted from one to 21 per cent of the flora.   The neutral types made up from 79 to 99 per cent of the organisms found in the gelatin.

The variation to be expected in bacterial analysis of gelatin is shown from calculations of the probable error of 100 plates made on the same sample.   The probable error of the average of duplicate plates based on 10-gram samples was 4.6 per cent; based on a one gram sample, the probable error was 8.5 per cent of the mean count.

If the samples included in this study represent a cross section of the gelatin available to the ice cream maker, the conclusion is warranted that gelatin pasteurized with the other ingredients of the mix, is a negligible source of bacteria in ice cream.

REFERENCES

AMBROSE, A. S. 1926 Ice Cream Review, **9**, 10, 132–136.

AYERS, S. HENRY. 1914 Ice Cream Trade Jour., **10**, 11, 38.

BAHLMAN, C. 1914 Ice cream studies in Cincinnati. Amer. Jour. Pub. Health, **4**, 1009.

BRANNON, J. M., AND TRACY, P. H. 1925 Gelatin as a source of bacteria in ice cream. Jour. Dairy Sci., **8**, 2, 115–126.

BURKE, A. D. 1923 Ice Cream Trade Jour., **19**, 3, 55–58.

ELLENBERGER, H. B. 1919 A study of bacteria in ice cream during storage. Cornell Univ. Agr. Exper. Station, Memoir 18.

GORDON, J. O. 1912 Ice Cream Trade Jour., **8**, 32–34.

GORDON, J. O. 1915. Ice Cream Trade Jour., **11**, 1, 24–26.

HALL, THOMAS, AND HOUTZ, R. L. 1923 Ice Cream Trade Jour., **19**, 6, 55–58.

HAMMER, B. W. 1912 Bacteria and ice cream. Ia. Agr. Exper. Sta. Bul. 134.

New York City Board of Health 1920 Proposed ice cream standards of New York City. Creamery and Milk Plant Monthly, **9**, 4, 56.

Oklahoma Experiment Station Biennial Report. 1923–1924. Cited in Exper. Sta. Rec., **53**, 177.

PARFITT, E. H. 1923a Some factors influencing quality in gelatin and the use of the fermentation test as an index of bacterial contamination. Jour. Dairy Sci., **6**, 4, 278–282.

PARFITT, E. H. 1923b Ice Cream Trade Jour., **19**, 8, 65–66.

SOMMER, H. H. 1927 Ice Cream Trade Jour., **23**, 1, 47.

TRACY, P. H. 1924 Ice Cream Trade Jour., **20**, 11, 75.

TURNBOW, J. D., AND MILNER, F. W. 1926 Ice Cream Trade Jour., **22**, 11, 134–136.

THE NITRITE TEST AS APPLIED TO BACTERIAL CULTURES

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It has been observed by one of us (G. I. W.) when making the nitrate reduction test on bacterial cultures that there is sometimes a fading or disappearance of the red color of the positive test. The test used was the sulfanilic acid-alpha-naphthylamine method, according to the Manual of Methods of the Society of American Bacteriologists. In extreme cases the color appeared only at the point of contact of the incoming naphthylamine solution and the test broth. The question arose as to whether or not the test could properly be called positive and the following investigation was made to find the cause of, and if possible a remedy for, this fading.

The fading was always noticed at a time when such organisms as *Escherichia coli*, *Aerobacter aerogenes*, *Proteus vulgaris* and the typhoid-paratyphoid group were being studied in the laboratory. Knowing that these organisms all produced more or less hydrogen sulfide, this compound was suspected as the bleaching agent. Accordingly a series of sixteen organisms were inoculated into lead acetate agar and nitrate broth tubes. Observations, after five days incubation, showed the following complete correlation between hydrogen sulfide production and the instability of the nitrite color (table 1). Where there was a very slight hydrogen sulfide production, the nitrite test color was permanent and where there was a large amount of hydrogen sulfide formed the nitrite test color faded immediately.

It was also found by using a series of sodium sulfide dilutions to which equal amounts of nitrite had been added that there was

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a marked weakening of the color (table 2). Hydrogen sulfide, however, simply prevents maximum color development, probably by destroying a part of the nitrous acid, but does not directly cause the fading under discussion. Since the nitrite color is due to the production of p-sulfo-benzene-azo-alpha-naphthylamine according to the equation:

$$NH_2\text{-benzene-}SO_3H + HNO_2 + \text{naphthalene-}NH_2 \longrightarrow SO_3H\text{-benzene}-N{=}N-\text{naphthalene-}NH_2 + 2H_2O$$
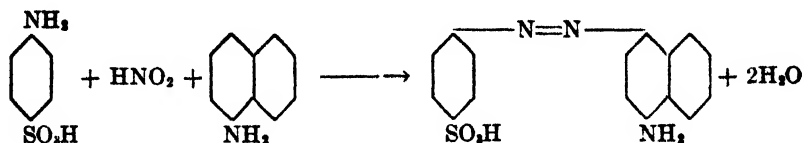
It would be expected that a reducing agent would destroy the color by producing a corresponding hydrazo compound. This would be operative in alkaline solution, but not under the conditions of the test.

Nevertheless, the correlation in table 1 shows hydrogen sulfide to be a factor, even though it is not the direct cause. In order to eliminate its effect, two samples of peptone were secured[1] which were devoid of cystine as shown by chemical analysis. Nitrate broth and lead acetate tubes were prepared with these peptones and the same sixteen organisms were inoculated into them. After five days incubation, the nitrate broths were tested with the standard reagents and none showed color fading, although some of the nitrite tests were so strong that a brown precipitate of the coloring matter soon developed. The lead acetate tubes were all negative. It was noted that with the strongly positive nitrite tests a single drop of naphthylamine solution gave a color which faded, but several additional drops produced a persistent color. This observation indicated that when a very small amount of the reagent was used the color faded; or, in other words, that in presence of a high concentration of nitrite the color fades. This view is borne out by the fact that where an ordinary nitrate broth tube shows fading, if a second tube of the same organism is diluted about one to five, thus diluting the nitrite formed, the second tube will not fade. These results all point to

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[1] We are indebted to the Illinois State Water Survey for these peptones.

the conclusion that, in the presence of hydrogen sulfide, such high concentrations of nitrite are produced that the normal color suffers complete destruction.

TABLE 1

Correlation between fading of nitrite color and hydrogen sulfide production

	NITRITE TEST TIME OF FADING [1]	H₂S PRODUCTION
1. *Escherichia coli*	1 minute	+
2. *Eberthella dysenteriae*	Permanent color	+ v. sl.
3. *Eberthella typhi*	½ minute	++
4. *Salmonella enteritidis*	½ minute	+++
5. *Salmonella pestis caviae*	1 minute	+++
6. *Salmonella suipestifer*	½ minute	+++
7. *Salmonella paratyphi*	1½ minutes	+ sl.
8. *Salmonella schottmulleri*	½ minute	+++
9. *Salmonella anatum*	½ minute	+++
10. *Salmonella pullorum*	1 minute	+
11. *Aerobacter aerogenes*	1½ minutes	+
12. *Proteus vulgaris*	½ minute	+++
13. *Alcaligines abortus*	Permanent color	+ v. sl.
14. *Proteus* X19	½ minute	+++
15. *Bacillus mesentericus*	Permanent color	+ v. sl.
16. Unknown spore former	2 minutes	+ sl.

TABLE 2

Effect of sodium sulfide on the nitrite color

MILLIGRAMS OF H₂S PER LITER OF SOLUTION	COLOR IN 10 MINUTES AS A PERCENTAGE OF THE CONTROL TUBE
0.0	100
5.0	95
9.6	90
14.4	85
19.2	70
24 0	50
28.9	40
33.6	20
38 5	10

Chemical tests have confirmed this and shown that an excess of of nitrous acid attacks the p-amino group of the coupled naphthyl-amine, the resulting diazonium salt breaking down with the for-

mation of the corresponding hydroxy-azo derivative; some further coupling may also occur. This view is supported by the fact that alpha-naphthol coupled with sulfanilic acid gives a brownish-yellow color strongly resembling the faded nitrite test. Spectrophotometrically the absorption maxima of the two solutions are coincident, though the faded nitrite test shows a slight secondary absorption attributable to the small additional coupling. Furthermore, fading in high nitrite concentrations was avoided by using naphthylamine in which the amino group had been protected by forming the mono-acetyl derivative. Coupling is slow, however, so that this remedy is not practical.

These facts suggest the use of some other compound for the coupling reaction. Of the common azo colors, the yellows and browns are unsuitable because they are difficult to detect in a normal yellow or brown nitrate broth. Dimethylaniline, however, gives a red color (free acid of methyl orange) very similar to the standard test. It possesses the advantages of stability and of giving a color which cannot fade because the amino group is protected. However, it will not detect less than one part of nitrite nitrogen in fifty million and probably would not be acceptable for this reason.   ·

As the object of this investigation was to improve the test now in use, nitrate tubes inoculated with the sixteen organisms were tested by other methods found in the literature. The Bismarck brown test as suggested by Griess (1878) and adapted by Preusse and Liemann (1878) does not contrast well with the normal color of the broth when only small amounts of nitrite are present. The Letts and Rea (1914) modification of the old Fresenius' iodide-starch method would be satisfactory if the reagent had greater permanency. The neutral red test suggested by Rochaix (1909) produces a very distinctive color, but it fades too rapidly. The benzidine method of Armani and Barboni (1911) was not tried as its yellow color with small nitrite concentrations would be a distinct disadvantage. The old Riegler reagent (1897) is probably not sensitive enough for general use, and the same criticism would apply to the indole test of Dane (1911) as recommended by Rosenthaler and John (1915).

None of the known tests mentioned above seem to offer a suitable substitute and the dimethylaniline lacks the desired sensitiveness. The sensitivity of any nitrite test used in bacteriological characterization affords a point for argument. Some investigators doubt the significance of exceedingly minute amounts of nitrite in cultures. For example, Harding (1910) favored the starch-iodide test because it was less delicate than the standard test. Conn and Breed (1919) on the other hand have emphasized the need for a very delicate test and the Committee on Bacteriological Technic of the Society of American Bacteriologists apparently are in agreement. As a result, dimethyl-alpha-naphthylamine was tried. It is a compound like dimethylaniline, but with a higher molecular weight which would

TABLE 3

Sensitivity of the standard and the proposed test

NITRITE DILUTION	STANDARD	PROPOSED
1:40 million	Distinct	Distinct
1:80 million	Distinct	Distinct
1:100 million	Faint	Faint
1:200 million	Faint	Very faint

All readings made after ten minutes.

give it a more intense color and consequently greater sensitivity. The results were so favorable that the recommendation that it be used in place of alpha-naphthylamine is offered without hesitation.

As obtained from the Eastman Kodak Company, dimethyl-alpha-naphthylamine is a slightly viscid liquid, specific gravity 1.016, with a faint kerosene odor and colorless except for a slight bluish fluorescence. The reagent, prepared by dissolving 6 cc. in one liter of 5 N acetic acid, is stable and apparently unaffected by exposure to light and air. The color produced with traces of nitrite is similar to the standard test. In high nitrite concentrations, the color does not fade because the amino group is protected. The colored compound does, however, tend to precipitate in high concentrations, but instead of turning brown or being entirely

destroyed as is the case with the standard test, enough of the dimethyl color remains in solution to give a dark red mixture which may be diluted with water to a clear red solution. The color production with very small amounts of nitrite is a little slower than the standard test, but the sensitivity of the two is alike (table 3). The sensitivity of the standard test was placed by Warington (1881) at 1:100 million though higher dilutions developed color on standing. Determinations have shown 1:100

TABLE 4

Comparison of the standard and the proposed test

	STANDARD TEST	PROPOSED TEST
1. *Escherichia coli*..........	Faded to yellow	Deep red; slight precipitate
2. *Eberthella dysenteriae*....	Brown precipitate	Deep red solution
3. *Eberthella typhi*..........	Faded to yellow	Deep red; slight precipitate
4. *Salmonella enteritidis*....	Faded to yellow	Deep red; slight precipitate
5. *Salmonella pestis caviae*..	Faded to yellow	Deep red: slight precipitate
6. *Salmonella suipestifer*...	Faded to yellow	Deep red; slight precipitate
7. *Salmonella paratyphi* ...	Faded to yellow	Deep red; slight precipitate
8. *Salmonella schottmülleri*.	Faded to yellow	Deep red; slight precipitate
9. *Salmonella anatum*......	Faded to yellow	Deep red; slight precipitate
10. *Salmonella pullorum*.....	Faded to yellow	Deep red; slight precipitate
11. *Aerobacter aerogenes*....	Nearly faded	Deep red; slight precipitate
12. *Proteus vulgaris*..........	Faded to yellow	Deep red; slight precipitate
13. *Alcaligines abortus*.......	Brown precipitate	Deep red solution
14. *Proteus* X19.....	Faded to yellow	Deep red; slight precipitate
15. *Bacillus mesentericus* ...	Brown precipitate	Deep red solution
16. Unknown spore former..	Nearly faded	Deep red; slight precipitate
17. Control..................	Very faint color	No color in 10 minutes

million in two minutes, 1:300 million in ten minutes and 1:500 million in thirty minutes. The proposed dimethyl compound shows 1:80 million in two minutes, 1:200 million in ten minutes and 1:400 million in thirty minutes. It is assumed that most bacteriologists allow a ten minute reaction period before taking a final reading on the nitrite test. Making allowance for the natural color of the broth, the standard test and the proposed modification are both sensitive to one part of nitrite nitrogen in one hundred million of solution. Sterile control tubes of nitrate

broth, when incubated for five days, not infrequently give faint positive tests with such delicate reagents.

Duplicate tubes of nitrate broth, made with ordinary sulfur containing peptone, were inoculated with the same series of organisms and tested for nitrite after five days incubation by both the standard and the new reagents (table 4). In the standard test the usual fading was noticed but in the proposed test it was not. In both tests the color appeared within thirty seconds after the addition of the reagents showing that with these organisms the tests are not only sufficiently delicate but that there is a margin of sensitivity that is adequate.

DISCUSSION

It has been shown that the fading in the standard nitrite test is caused by an excess of nitrite. In a great many cases the color fades almost instantly and could very easily be overlooked by the busy laboratory worker making a number of tests. It has also been shown that in cultures of hydrogen sulfide producing bacteria this fading is more marked. This condition is not due to the organism itself but to the action of the hydrogen sulfide formed. The trouble is alleviated by the use of a sulfur free medium. A new test is recommended that does not have the disadvantages of the standard test.

CONCLUSIONS

1. In high nitrite concentrations, the color resulting in the standard nitrite test is destroyed on account of the destruction of the amino group of the naphthylamine.

2. The production of high nitrite concentrations is favored by the use of peptones containing sulfur and, conversely, small amounts of nitrous acid escape detection in the presence of hydrogen sulfide.

3. Dimethyl-alpha-naphthylamine gives a stable reagent of high sensitivity and produces a permanent nitrite color.

4. It is recommended, therefore, in place of alpha-naphthylamine.

REFERENCES

ARMANI AND BARBONI   1911   Chem. Ztg., **34, 994**.

CONN AND BREED   1919   Jour. Bacteriol., **4**, 267–290.

DANE   1911   Bull. Soc. Chim. **9, 354–355**.

GRIESS   1878   Ber., **11, 624–627**.

HARDING   1910   New York Agr. Expt. Sta., Techn. Bull. 13.

JOHN   1915   Apoth. Ztg., **30, 265–266**.

LETTS AND REA   1914   Analyst, **39, 350–352**.

PREUSSE AND LIEMANN   1878   Ber., **11, 626–637**.

RIEGLER   1897   Z. Anan. Chem., **35**, 677–678; **36**, 306–307; Pharm. Centralhalle, **38**, 223.

ROCHAIX   1909   Compt. rend. Soc. Biol., **66**, 171.

WARINGTON   1881   Jour. Chem. Soc., **39**, 229–234.

AN ANAEROBE ISOLATED FROM DENTAL CARIES

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In the literature concerning the bacteriology of the mouth a certain group of organisms has heretofore been dealt with in an indefinite manner. I refer particularly to *Bacillus buccalis-maximus* (Miller), *Leptothrix buccalis* (Robin), and *Leptothrix buccalis* (Vignal). Varying characteristics have been attributed to these organisms by different authors.

Robin, basing his observations on direct smear preparations, gives only a vague description of *Leptothrix buccalis*, but he does point out that they are long unarticulated threads. Vignal studied an organism, which he called *Leptothrix buccalis*, in pure culture. Though his organism formed threads, these threads were definitely articulated. Miller describes *Bacillus buccalis-maximus* as a rod, 2 to 10 micra long. These usually occur in long articulated threads which tend to form tufts. Miller was not able to cultivate this organism, but from his studies of smear preparations he concluded that it was different from the leptothrices.

Among other forms found in direct smear preparations taken from advanced carious lesions in teeth an unusually long, thick Gram positive organism is invariably present. When it appears as a chain of rods, each rod measuring some 10 micra in length, the organism is spoken of as *Bacillus buccalis-maximus.* Should it appear as a single thread from 20 to 100 micra long it is called *Leptothrix buccalis* (Robin). If the long thread is definitely articulated the organism is considered *Leptothrix buccalis* (Vignal).

This paper describes an organism which when grown in pure

culture coincides with each of the varying conceptions listed above.

Blood agar plates were streaked with debris taken from advanced dental caries. For this purpose Douglas medium of pH 7.6 with 2 per cent agar and 5 per cent rabbit blood was found satisfactory. The plates were placed in an anaerobic jar (McIntosh) and incubated at 37°C. for forty-eight hours.

In forty-eight hours a profuse foul-smelling growth is obtained. This growth consists largely of coccoid forms, very many Gram-negative bacilli, and spirilla. Isolated colonies of the desired organism are rarely found on these first plates. However, a careful examination of such a culture will reveal a few extremely delicate filaments which radiate from under the mixed growth out on to the clear agar. A second plate inoculated from such a filament will yield a luxuriant growth of the organism in pure culture.

Twelve strains were isolated from dental caries. It was noticed that while the organism was readily isolated from old open lesions it was rarely obtained from initial caries of enamel. Such findings may have been due to chance. No attempt has been made to determine the incidence nor the significance of this organism in dental caries.

The organism is a Gram-positive rod, measuring 0.3 micron in width, and may vary from about 8 micra to an extremely long filament extending across the entire field. The organism is easily stained with methylene blue, carbol fuchsin, and the usual basic aniline dyes.

The rods may occur singly or in chains of 3 to 8 or more cells. The isolated rods are somewhat tapered, though, not infrequently, clubbed and swollen ends occur. Figures 6 and 8 illustrate the striking resemblance of these organisms to *Bacillus buccalis-maximus*. Figure 3 shows the characteristic swellings.

When these organisms grow out into threads they may be articulated or uninterrupted in their entire length, as can be seen from figure 4.   The thread forms are frequently wavy and all of the strains have the unusual tendency to form coils with turns of mechanical uniformity (figs. 4 and 5).   In this latter respect the organism resembles Weible's *"Vibrion aus Nasenschleim,"* (fig. 7).   Very often two organisms are seen wound around each other in cable fashion.   Such forms may be seen in figures 1 and 2.

CULTURAL CHARACTERISTICS

For isolating this organism, Hartley's modification of Douglas's medium with 2 per cent agar and 5 per cent blood, is very satisfactory.   It seems to be the medium best suited for the growth and cultivation of these forms.   On plain agar or meat infusion agar little or no growth occurs.   In some instances a slight growth was obtained on the first transfer to meat infusion agar, but it was not possible to secure growth after the second transfer to media which did not contain blood or hydrocele fluid.

Gelatin, casein digest sodium oleate agar, whey agar, milk, brain broth, and glucose meat infusion broth are unsatisfactory.

With Loeffler's serum and ascitic fluid agar, a meager growth may be obtained.   Hydrocele fluid agar may be used with some satisfaction, but blood agar is excellently suited for this organism. Growth in broth was obtained only with difficulty.   All tests for the production of indol were negative.

On blood agar the colonies vary from 1 to 3 mm. in diameter. The smaller colonies are slightly raised, circular, and have discrete borders.   The older colonies are much larger, measuring from 3 to 4 mm. in diameter.   In the middle of each colony is a whitish papule from which the colony substance seems to radiate.   Immediately around this peak the matrix is uniformly dense and of a greyish tint.   Toward its periphery the colony breaks up into hair like filaments which terminate in three or four tufts.   These tufts generally take on an S-shaped curve giving to the colony a whirl appearance.   Such typical colonies often appear within forty-eight hours.

The pure cultures have a slightly musty odor but are not foul.

The organism is non-motile. It is not pathogenic. Large amounts of heavy suspensions in saline were injected into guinea pigs both intraperitoneally and subcutaneously with no untoward results.

The optimum temperature for growth is 37°C. A scant growth may be obtained at 40°C. No growth takes place at 18° to 20°C. It does not hemolyze blood and produces no pigment. No spores have been observed.

ACTION ON CARBOHYDRATES

Brom cresol purple in saturated alcoholic solution was added to Douglas agar in sufficient amount to give to the medium a fairly deep blue color. This was put up in 25 cc. amounts and autoclaved. Two per cent aqueous solutions of the carbohydrates to be used were passed through a Seitz filter and then added under sterile conditions to the agar in 25 cc. amounts. In this manner the sugars were not subjected to heating and the final carbohydrate content was 1 per cent. To insure growth a drop of blood was added to each slant.

All strains studied produced acid without gas from glucose, levulose, mannose, lactose, sucrose, raffinose, arabinose, xylose, mannitol, dulcitol, sorbitol, and inositol. Acid was not produced by the growth of the organism in the film of blood on the agar slant when no carbohydrate was added.

COMMENT

The organism described above might well be classified as a *Leptothrix*, if such a genus be valid. As seen in the fresh material from which it is isolated the organism is filamentous. In culture it grows out into long non-branching threads, though indeed unusually long bacillary forms occur. Morphologically it tallies very well with what previous observers have ventured to think of as leptothrices. In a recent paper Varney describes several strains as *Bacillus fusiformis*. One or two of these strains resemble the organism which I have described. Pending further studies, however, I shall reserve for a future paper the classification of this organism.

SUMMARY AND CONCLUSIONS

Twelve strains having similar morphological and cultural characteristics were isolated from dental caries. They are strictly anaerobic and grow best on blood agar. Though they utilize carbohydrates to produce acid, their status as an etiological factor in dental caries is extremely doubtful. They differ from the leptothrix isolated by Vignal in that Vignal's organism grew very well aerobically on the ordinary laboratory media.

The pleomorphism of this organism is very likely responsible for the differences of opinion advanced by early workers who were not able to observe the organism in pure culture.

REFERENCES

HARTLY, P. 1922 The value of Douglas's medium for the production of diphtheria toxin. Jour. Pathol. and Bacteriol., **25**, 479.

McINTOSH AND FILDES 1916 A new apparatus for the isolation and cultivation of anaerobic microörganisms. Lancet, **1**, 768.

MILLER, W. D. 1898 Microörganisms of the Human Mouth. S. S. White, Philadelphia, p. 73.

ROBIN 1847 Des Végétaux qui Croissent sur les Animaux Vivants. Paris, p. 42.

VARNEY 1927 The serological classification of fusiform bacilli. Jour. Bacteriol., **13**, 275.

VIGNAL 1886 Recherches sur les microorganismes de la bouch. Archiv. d. Physiol. norm. et pathol., p. 325.

WEIBLE, E. 1887 Untersuchen über Vibrionen. Centralbl. f. Bakteriol., etc., I abt., orig., **11**, 465.

PLATE 1

FIG. 1. Smear from three-day culture on blood agar.

FIG. 2. Braided filaments from three-day culture on blood agar.

FIG. 3. Swelling at end of filament from forty-eight-hour culture on blood agar.

FIG. 4. Smear from forty-eight-hour culture on blood agar, showing spirals and coils.

FIG. 5. Typical coil from forty-eight-hour culture.

FIGS. 6 AND 8. Forms resembling rods and chains of so-called *Bacillus buccalis-maximus*, from forty-eight-hour culture on blood agar.

FIG. 7. Copy of figure in Weible's paper showing coils of filamentous organism from nasal mucus.

PLATE 1

Nasenschleim -Vibrio aus Agar

(Jay An Anaerobe Isolated from Dental Caries)

SIMPLE METHOD OF PRODUCING ANAEROBIC CONDITIONS

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A simple method of producing anaerobic conditions is to set a small tube filled with pyrogallic acid solution so that its open capillary tip discharges into a small beaker containing the proper amount of KOH solution and evacuate it in a closed system— as shown in picture and sketch.
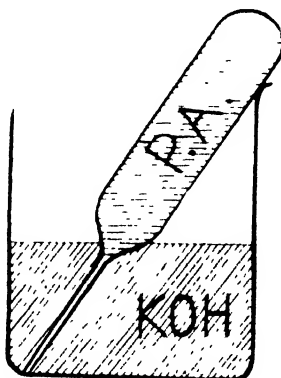
FIG. 1

As the pyrogallic acid is not mixed with the KOH solution until the jar is exhausted, waste is avoided and only a small amount, which may readily be calculated is needed. If hydrogen or nitrogen is introduced after evacuation, the amount of mixture drawn back into the tube must, of course, be taken into consideration. The pyrogallic tube itself is filled by setting it into a

beaker containing pyrogallic acid and evacuating. The air escapes from it and on releasing the vacuum the pyrogallic acid is drawn into the tube.
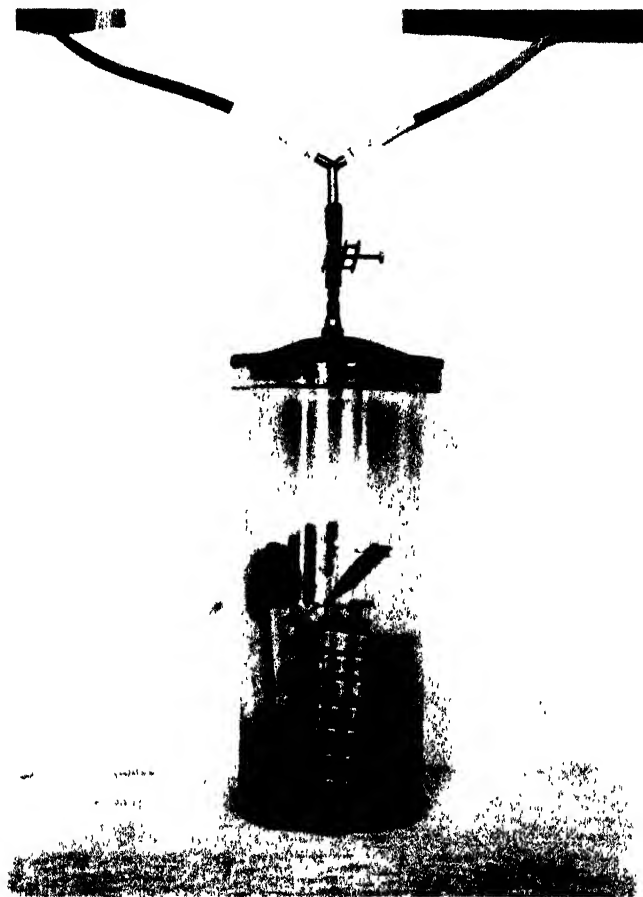
FIG. 2

For quick reference a diagram giving the amount of 22 per cent pyrogallic acid solution necessary for the absorption of oxygen per liter under various pressures in mm. Hg is attached. The 10 to 60 per cent KOH solution (depending on the rate of O_2 absorption desired), with which it is to be mixed, should be six

times that amount.   The diagram is based upon the absorption of 12 cc. of O_2 in air at atmospheric pressure by 1 cc. of the pyrogallic acid KOH mixture.

FIG. 3.   AMOUNT OF PYROGALLIC ACID SOLUTION NEEDED TO ABSORB THE O_2 IN AIR PER LITER AT VARIOUS PRESSURES

REFERENCE

TREADWELL: Analytical Chemistry, vol. ii, Quantitative Analysis, 1924 edition, p. 653.

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THE CHINA BLUE-AURIN-CELLULOSE MEDIUM FOR THE PHYSIOLOGICAL STUDY OF CELLULOSE DESTROYERS

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INTRODUCTION

In previous papers (Sanborn (1926, 1927)) the author has shown that the growth and physiological efficiency of cellulose destroyers are markedly influenced by the presence of a so-called essential food factor in the medium. This factor, present in sterile, unheated plant tissue, in extracts from the seedlings of various crops, and as a synthetic product of the microbial cell, plays an important part in natural processes of cellulose destruction, as, for example, in the decomposition of green manure.

Microbial associations, too, exert a significant influence upon the rate of cellulose fermentation and the effect produced seems to be intimately related to the provision of essential food substances. A similar conclusion regarding the nature of association has been reached by other investigators, Kent (1924), Davis (1921)). The essential food factor causes the cellulose destroyer to attain a state of maximum efficiency and the process of cellulose decomposition in the laboratory may be accomplished with an efficiency which even surpasses that commonly observed in nature.

For details in technique and method, reference should be made to the previous papers.

THE CHINA BLUE-AURIN MEDIUM FOR THE ISOLATION AND INVESTIGATION OF CELLULOSE DESTROYERS

Based upon the physiological proclivities of the cellulose-decomposing organisms studied and the successful application of

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the H-ion technique as a criterion of the rate of decomposition, a medium has been developed and described (Sanborn (1926)), which furnishes a convenient method of testing the influence of essential food substances and association upon the physiological efficiency of cellulose-destroyers. A modification is recommended in this paper which offers a wider range of usefulness. In the previous work it was found desirable to employ a natural, unaltered form of cellulose. Raw cotton, which was selected, proved highly satisfactory. The seed fibers of the milkweed, a form of cellulose used by the author at present, is also yielding favorable results.

In the present investigation an attempt has been made to prepare a medium using raw cotton, precipitated according to the method of McBeth (1916). The dissolution of the cotton in copper-ammonium solution was carried on as rapidly as possible by constant shaking, followed immediately by precipitation and washing.

The composition of the liquid medium is as follows:

	cc.
Precipitated raw cotton	500
Basic nutrient solution[1]	500
China blue-aurin indicator[2]	10

To prepare the solid medium, 1.5 per cent agar is dissolved in the nutrient solution, then the cotton is added, and, finally, with constant stirring, the indicator. The medium becomes of a bright red color which is not affected by the autoclaving which follows. The pH is approximately 8.40. Plates may be poured in the usual way, and the organisms which decompose cellulose with the production of acid, form blue zones about the growth, or the growth itself may take up the stain. Besides facilitating the isolation of cellulose destroyers the medium is useful for determining physiological efficiency. Either the liquid or solid medium will yield satisfactory results, but in the majority of the experiments the

[1] Referred to previously; described in first paper of series.

[2] Indicator is obtained by mixing equal parts of 0.5 per cent aqueous solution of China Blue with 1.0 per cent solution of rosolic acid in 95 per cent alcohol. (See Jour. Bacteriol., (1926), 12, 351.)

author has employed the latter in Petri plates, making parallel streaks and treating one-half of the medium in the plate with the accessory factor, leaving the other half as a control. The incubation temperature was 25°.

If the liquid medium is tubed, autoclaved and inoculated with the organism to be tested, the essential food element may be added by introducing into series of tubes varying amounts of extract. Positive results are indicated by the gradual fading of the bright red color, the medium becoming an intense blue within a few days. A comparison of such tubes with the controls will thus reveal visible evidences of cellulose decomposition.

In the physiological investigation of cellulose fermentation by *C. folia*, the author has secured marked stimulative effects with the following materials:

1. Sterile, unheated plant extracts (alfalfa, barley, buckwheat, clover)
2. Extracts prepared from cells of *B. mycoides*, *B. subtilis*, *B. cereus*, cellulose destroyers, and yeast
3. Suspensions of living *Azotobacter* cells

CONCLUSION

The present article is a preliminary report on the use of the China blue-aurin-cellulose medium. Further work is in progress at this laboratory.

REFERENCES

Davis, D. J. 1921 Jour. Infect. Dis., **29**, 186.
Kent, S. S. S. 1923–1924 Jour. Hyg., **22**, 52.
McBeth, I. G. 1916 Soil Sci., **1**, 438.
Sanborn, J. R. 1926 Jour. Bacteriol., **12**, 1.
Sanborn, J. R. 1926 Jour. Bacteriol., **12**, 343.
Sanborn, J. R. 1927 Jour. Bacteriol., **13**, 113.

A COMPARATIVE STUDY OF SIX DIFFERENT STRAINS OF THE ORGANISM COMMONLY CONCERNED IN LARGE-SCALE PRODUCTION OF BUTYL ALCOHOL AND ACETONE BY THE BIOLOGICAL PROCESS

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A survey of the literature reveals the fact that as far back as 1876 butyl alcohol was recognized as a metabolism product of certain types of bacteria and moulds. Since the pioneer work of Fitz and Pasteur, some twenty-five writers have described organisms which produce butyl alcohol from carbohydrate material. The biological production of acetone was not noted until later (1904), but it is highly probable that all the forms which produce butyl alcohol likewise form acetone, and that the majority of those described in the literature belong to a single species. The types which have been studied more recently have been shown to be capable of considerable variation, depending upon the nutrient medium employed, age of culture, and respiratory environment. The particular organism which is being used on a commercial scale has been given a variety of names which cause confusion regarding the identity of the species. We would urge the general acceptance of the name *Clostridium acetobutylicum*, Weizmann, as being descriptive and in compliance with the rules and nomenclature of the Society of American Bacteriologists.

In the present experimental work it has been our aim to make a comparative study of various strains of butyl alcohol and acetone producing organisms, both newly isolated and old known stock strains. The investigation was stimulated in part by the fact that this laboratory was made a temporary repository

for an authentic culture which had been employed for some time in the production of solvents.

ISOLATION OF NEW STRAINS

When this work was undertaken, only the single strain of the butyl organism was available for study. Four others were isolated later, and finally one more strain was received from another laboratory.

The isolation method was similar to that described by Weizmann (1919). To test tubes containing 10 cc. of sterile corn mash (5 per cent) were added small amounts of the original test substances. The test tubes were heated in a boiling water bath for 45 seconds, then promptly cooled in running water. After incubation for from twenty-four to thirty-six hours, those tubes which showed the most pronounced amylolytic action and gas production were selected as probably containing the butyl-acetone organism. After from eight to ten days further incubation, the tubes were again heated as before and the contents plated on agar in various dilutions. Within two days typical polyhedral colonies, when present, were picked from the plates and inoculated into fresh tubes of mash.

Considerable time was devoted to a study of different solid media. The following combination invariably gave the best results.

Malt extract..	5 grams
Agar..	20 grams
Gelatin...	10 grams
Saturated solution $CaCO_3$	1000 cc.

Following autoclave heating, and the filtering off of the dark precipitate which forms, the medium is tubed and sterilized.

As the organism is a strict anaerobe, special methods of culturing had to be employed. The Laborius-Veillon method involving the use of long glass shake tubes was at first tried. This procedure gave very large and perfect colonies, but experience proved that there was considerable danger of contamination during the slicing of the medium.

The anaerobic culture method described by Krumwiede and Pratt (1913) was much more convenient and safe. After inoculation, the medium was poured into the inverted cover of a sterile Petri dish and on the surface of the still liquid medium was floated the other section of the Petri dish. The two parts of the plate are readily separated after incubation, and the gum-like colonies picked with a platinum needle through a *very thin* layer of medium.

Strain St, the original solvents culture, had been isolated in 1919 from barley grown in Pennsylvania. It was found to be quite active after a few transfers in 5 per cent mash. This strain had been carried in mash since its isolation and had been frequently heated and subcultured. It gave a brisk fermentation and a good yield of acetone and butyl alcohol from the beginning.

Strain K was isolated in the laboratory in April, 1924, from a putrefying clam. It gave a rather feeble but typical butyl fermentation upon isolation.

Strain M was isolated in May, 1924, from corn meal bought at a local market. When freshly isolated, the fermentation of corn mash was brisk but in no way comparable to that of *Strain St*.

Strain So was obtained in pure culture from Connecticut garden soil in October, 1924. At the outset it gave such feeble fermentation that it was thought to be an organism of a different species. Continued cultivation increased its activity.

Strain B was isolated in the fall of 1925 from barley obtained in the market. Its fermentative powers were quite strong after a few transfers.

Strain S was obtained from another laboratory and was highly developed as an acetone and butyl alcohol producer when received. Its date of isolation and origin are unknown.

Although approximately 75 samples of material were used in attempting to isolate the butyl-acetone bacillus, in only 4 cases was it finally recovered in a pure state. Attempted isolations from rye, wheat, millet, hemp, bird seed, rancid butter, sewage, rotting potatoes and feces were unsuccessful.

While many of the grains and most of the soils investigated

gave butyl or butyric fermentations, the lack of strongly marked diastatic action showed that if the organism desired was present it was far outnumbered by other forms, and that isolation would be practically impossible.   In such cases repeated heating and reculturing failed to bring it into view.   It is possible that in many cases associated bacteria so masked the activities of the butyl bacillus that it could not be recognized as such.

All six strains were identical in the various qualitative tests. They differed greatly, however, in intensity of reactions, notably in their ability to produce n-butyl alcohol and acetone from carbohydrate material.

A complete bacteriological description of the organism could not be found in the literature.   The following descriptive outline was prepared according to the chart of the Society of American Bacteriologists.

<div align="center">DESCRIPTIVE CHART</div>

Source.   Connecticut garden soil.
Date of isolation.   October, 1924.
Name.   *Clostridium acetobutylicum*, Weizmann.[1]

<div align="center">*Morphology*</div>

Vegetative cells.   Medium: 5 per cent corn mash.   Temperature: 37°.   Age: Twenty-four hours.   Form: Short rods.   Arrangement: Single.   Limits of size: 2μ to 5μ by 0.4μ to 0.6μ.   Size of majority: 3μ by 0.5μ.   Ends: Rounded.   Capsules: Present in old glucose peptone cultures.   Stained with carbol fuchsine.

Sporangia.   Present.   Medium: 5 per cent corn mash.   Age: Forty-eight hours.   Temperature: 37°C.   Form: Spindle.   Limits of size: 4μ to 6μ by 1μ to 1.2μ.   Size of majority: 4μ by 1μ.

Endospores.   Present.   Location: Sub-polar.   Form: Cylindrical to ellipitical.   Limits of size: 1μ to 1.6μ by 0.8μ.   Size of majority: 1μ by 0.8μ.   Wall: Thick, non-adherent.

Motility.   Motile.   Flagella: Present, peritrichiate.

Irregular forms.   Present in old glucose containing media: cuneate.

Staining reactions.   Methylene blue: Deep blue.   Other stains: Stains deeply.   Gram stain: Positive.   Lugol's iodine: Granulose.

[1] This name has also been proposed recently by McCoy, Fred, Peterson and Hastings (1926).

Cultural characteristics

Aerobic agar stroke.   No growth.

Gelatin stab.   Very slow, limited growth.   Line of puncture: Plumose in depth of medium.   Liquefaction: Slight.

Malt extract gelatin agar anaerobic tubes.   Polyhedral granular colonies, edge entire, 2 mm. in diameter in five days.   Cream white in color.   Very viscid.   Gas splits the medium.

TABLE 1

Showing the fermentation of sugars, etc., in peptone water containing pulped filter paper

	GROWTH	ACID	SOLVENTS
Glucose.................................	++	++	++
Lactose.................................	++	++	++
Sucrose.................................	++	++	++
Levulose................................	++	++	++
Galactose...............................	++	++	++
Mannose................................	++	++	++
Arabinose..............................	++	++	++
Xylose.................................	++	++	++
Calcium lactate.........................	−	−	−
Starch..................................	++	++	++
Pectin..................................	++	++	++
Inulin..................................	+	++	+
Mannitol...............................	++	++	+
Glycerol................................	−	−	−
Glycogen...............................	++	++	+
Dextrin................................	++	++	++
Rhamnose..............................	−	−	−
Melezitose.............................	++	++	−
Trehalose..............................	−	−	−
Melibiose..............................	−	−	−

Nutrient broth.   No growth.

Potato.   Abundant in anaerobic tube.   Form of growth: Slimy, cream-colored, opaque.   Elevation: Flat.   Odor: Butyl.

Peptone starch paste.   Growth: Rapid.   Diastatic action: Rapid, complete

Litmus milk.   Coagulation: Rapid.   Coagulum: Peptonized.   Reaction: Acid, finally reduction.

Nitrogen.   Secured from protein and peptone.

Best medium for growth. 5 per cent corn mash.

Best medium for colony formation. Malt extract gelatin-agar.

Quick differential test. Diastatic activity, odor.

Pathogenicity. Non-pathogenic (rabbit, guinea pig).

Loss of vitality on culture media. Viable for years.

Acids produced. Acetic, butyric and lactic.

Alkali produced. None.

Alcohols produced. Ethyl and butyl.

Aldehyde. Slight.

Acetone. Strong.

Ferments present. Peptase, diastase, pectase, glucase, lactase, invertase, inulase (some of which are not secreted into the medium).

Tolerance to acids. Great.

Tolerance to alkali. Slight.

Resistance to drying. Very resistant.

NITROGEN REQUIREMENTS OF CL. ACETOBUTYLICUM

Carbohydrate metabolism has been quite thoroughly covered in other researches. On the other hand, such data as the following on available nitrogen sources have not been presented.

The composition of the elementary medium employed in this work was as follows:

K_2HPO_4	0.5 gram
KH_2PO_4	0.5 gram
$MgSO_4$	0.2 gram
NaCl	0.01 gram
$FeSO_4$	0.01 gram
Wheat starch	30.00 grams
H_2O distilled	1000.00 grams

Pulped filter paper was added to give "body" to the medium, and thus to aid in establishing a favorable (anaerobic) condition. Starch was used for carbohydrate, as diastatic action furnishes a ready index of growth. The nitrogen-free medium was tubed in 30-cc. amounts in large test tubes and the nitrogenous materials to be investigated were added in varying concentrations. After sterilization, the tubes were inoculated with a saline suspension of active solvents-producing bacilli which had been grown in maltose-peptone broth, centrifuged and thoroughly washed.

In the first set-up, the added nitrogen was in the form of protein. The object of this experiment was to determine whether any particular protein was more suitable for fermentation than others. The corn protein fractions were prepared in the laboratory; the zein was removed by alcoholic extraction, and the globulin fraction separated by extraction with salt solution and subsequent precipitation by dialysis. The ovalbumin and egg-meat proteins were added in the desiccated state. The total protein content of 5 per cent corn mash is about 0.4 per cent. This figure gave the basis for the concentrations used. In each

TABLE 2

Showing the availability of different proteins as sources of nitrogen

	TUBE 1	TUBE 2	TUBE 3
Zein, 0.01 per cent..........................	+++	+++	+++
Zein, 0.4 per cent...........................	+++	+++	+++
Zein, 1 0 per cent...........................	+++	+++	+++
Globulin, 0 01 per cent......................	++	++	++
Globulin, 0.4 per cent.......................	+++	+++	+++
Globulin, 1.0 per cent.......................	+++	+++	+++
Egg-meat, 0.01 per cent.....................	+++	+++ ·	+++
Egg-meat, 0.4 per cent......................	+++	+++	+++
Egg-meat, 1.0 per cent......................	+++	+++	+++
Ovalbumin, 0 01 per cent....................	+++	+++	+++
Ovalbumin, 0.4 per cent.....................	+++	+++	+++
Ovalbumin, 1.0 per cent.....................	+++	+++	+++
Control (no. N_2)..............................	−	−	−

instance where growth occurred, a portion of the contents of that particular tube was used to inoculate another tube of the same medium. This was repeated for three tubes. (See table 2.)

It is evident from this experiment that various types of protein constitute available sources of nitrogen. All gave active fermentations, including the protein fractions from the corn. The tubes which contained the zein seemed to be slightly more active than the others.

The second experiment was made in order to determine the availability of various amino acids and related compounds. The following substances were used: Glycocol, proline, valine,

cystine and aspartic acid. A control, free of all nitrogen, and a control containing peptone, were included. (See table 3.)

This experiment would indicate that the butyl bacillus is unable to utilize simple amino acids of the above types, when used as the only source of nitrogen.

Experiment III included miscellaneous nitrogenous compounds —purine substances, urea and inorganic salts. (See table 4.)

None of the inorganic salts employed here supported growth. This observation is not in accord with certain claims that the butyl organism is capable of deriving nitrogen from ammonium salts.

TABLE 3

Giving results with amino acids as possible sources of nitrogen

	TUBE 1	TUBE 2	TUBE 3
Glycocol, 0.01 per cent	−	−	−
Glycocol, 0.5 per cent	−	−	−
Proline, 0.1 per cent	−	−	−
Proline, 0.5 per cent	−	−	−
Valine, 0.01 per cent	−	−	−
Valine, 0.5 per cent	−	−	−
Cystine, 0.01 per cent	−	−	−
Cystine, 0.5 per cent	−	−	−
Aspartic acid, 0.1 per cent	+	−	−
Aspartic acid, 0.5 per cent	−	−	−
Peptone (control) 0.5 per cent	+++	+++	+++
Control (no. N_2)	−	−	−

Small amounts of cultures of various types of organisms, viz., *E. prodigiosus, Staph. aureus, Proteus vulgaris*, etc., scraped from agar slants, when added to the nitrogen-free medium, supported an especially vigorous development of the butyl organism. The results were the same, whether the nitrogen-bearing material was either dead or alive.

Attempts were also made to grow a nitrogen-fixing species (*Azotobacter croöcoccum*) in nitrogen-free media, in order to produce sufficient combined nitrogen to support the butyl organism when later introduced. All such attempts were without success.

In many cases the unsuitable nitrogenous substances could be converted by another bacterial species into a form capable of supporting the butyl fermentation. For instance, a medium containing nitrogen only in the form of glycocol was inoculated with *Staphylococcus aureus*. After forty-eight hours incubation, the tubes were inoculated with the butyl bacillus, which then underwent abundant development.

It is evident from these experiments on the nitrogen requirements that the solvents-producing organism is dependent upon

<div align="center">TABLE 4</div>

	TUBE 1	TUBE 2	TUBE 3
Nucleic acid, sample "b," 0.05 per cent	−	−	−
Nucleic acid, sample "b," 0.5 per cent	−	−	−
Urea, 0.05 per cent	−	−	−
Urea, 0.5 per cent	−	−	−
Uric acid, sample "a," 0.05 per cent	−	−	−
Uric acid, sample "a," 0.5 per cent	−	−	−
Ammonium lactate, 0.05 per cent	−	−	−
Ammonium lactate, 0.5 per cent	−	−	−
Ammonium sulphate, 0.05 per cent	−	−	−
Ammonium sulphate, 0.5 per cent	−	−	−
Ammonium nitrate, 0.05 per cent	−	−	−
Ammonium nitrate, 0.5 per cent	−	−	−
Sodium nitrate, 0.05 per cent	−	−	−
Sodium nitrate 0.5 per cent	−	−	−
Sodium nitrite, 0.05 per cent	−	−	−
Sodium nitrite, 0.5 per cent	−	−	−
Peptone control	+++	+++	+++
Nitrogen free control	−	−	−

highly complex compounds—proteins and peptone, for growth, utilizing these through the agency of a very active proteolytic enzyme. In the presence of such compounds, simpler nitrogenous substances may or may not be also available for assimilation.

<div align="center">SEROLOGICAL RELATIONSHIPS</div>

For the production of the antigens, both for inoculation purposes and for use as test antigen, the several strains were

grown in 100 cc. Erlenmeyer flasks containing 50 cc. of a medium of the following composition:

K_2HPO_4...	1.0 gram
KH_2PO_4...	1.0 gram
$MgSO_4$..	0.2 gram
NaCl...	0.01 gram
$FeSO_4$..	0.01 gram
Peptone..	5.0 grams
Maltose..	20.0 grams
H_2O distilled..	1000.0 cc.

Small bits of filter paper were added to the filtered medium and after forty-eight hours incubation of the inoculated flasks they were freed from the filter paper, the bacterial suspension was

TABLE 5

Antiserum for strain K

	1:200	1:400	1:600	1:800
Antigen K..........................	+++	++	++	+
Antigen So..........................	+++	+	+	−
Antigen St..........................	+++	++	+	−
Antigen B..........................	+++	++	+	+
Antigen M..........................	++	+	+	−

centrifuged and the supernatant liquid poured off. Following one washing with saline solution, and re-centrifugalization, the cells were suspended in a rather concentrated state in carbolized saline solution. For use as a vaccine, this suspension was diluted to an opacity equal to 4.0 on the McFarland nephelometer scale and heated at 58° for thirty minutes. For use as antigen in the agglutination tests, 0.75 (neph. scale) suspensions were employed.

Antisera for strains K and B were prepared by the intravenous injection of rabbits with 0.5 cc. doses of the bacterial suspension at 2 day intervals over a period of ten days. The animals were bled on the 21st day after the first injection and the sera prepared. The usual macroscopic technique was employed.

Serum prepared from strain K was used against antigens K, So, St, B and M. (Strain S was not available at this time.)

The experiment represented in table 5 was repeated several times with similar results. The controls were negative.

In the following experiment, antiserum prepared from strain B was employed. Its action on antigens of all strains was tested. (See table 6.)

The cross-agglutination between the different strains as evidenced in these tables would confirm the species identity of the strains under observation. Moreover, the reaction of the different strains with the two sera in somewhat different degrees would indicate that the various strains were of different origin, and that none were reisolations of a single existing strain.

TABLE 6

Antiserum for strain B

	1:200	1:400	1:600	1:800	SERUM FREE CONTROL	NORMAL SERUM CONTROL
Antigen B................	+++	+++	++	++	−	−
Antigen M................	++	++	−	−	−	−
Antigen So................	+++	+	−	−	−	−
Antigen K................	+++	++	−	−	−	−
Antigen St................	+++	++	−	−	−	−
Antigen S................	+	+	−	−	−	−

METHODS OF ANAEROBIC CULTURE

While experimenting with butyl fermentations several modifications were made in the customary methods of handling the cultures and of analyzing them for acetone and butyl alcohol.

In order to transform dormant spore material into an actively fermenting culture, strict anaerobic technique must be employed. The method described by Gruber (1887) has been generally employed in the commercial preparation of cultures. This technique was used in the early part of this work. Long test tubes, 12 inches by ⅝-inch, are slightly constricted in the middle by heating in order to prevent the cotton plugs from slipping down into the mash. A 10-cc. quantity of mash is then introduced into each tube by means of a long pipette, and the tubes plugged and sterilized. After inoculation with from 0.5

to 1 cc. of a sporulated culture, and pushing the plug as far as the constriction with a glass rod, the tube is connected with a vacuum pump and the air rapidly exhausted (gentle boiling at room temperature). The plug is then heated to charring through the glass and the tube slowly drawn out and sealed at a point just above the plug.

The method of Avery and Morgan (1923) for anaerobic culture was used with success. "Artificial peroxidase" is prepared by dissolving gum arabic in a warm solution of ferrous sulphate and precipitating with 95 per cent ethyl alcohol. The precipitate is filtered off and added in small quantities (i.e., a small loopful) to ordinary test tubes containing 10 cc. of the spore-inoculated mash. Upon incubation, the spores develop in the presence of apparently unlimited oxygen. Tests were made which proved that the catalase may be autoclaved with the medium or alone without impairing its efficiency. It loses its value, however, very rapidly with age, so that a new lot must be made up every two or three days.

The use of aerobic forms for stimulating anaerobes to growth under conditions of normal oxygen tension has often been resorted to. This method was employed with decided advantages over those just described. By the simultaneous inoculation of sterile mash with spores of the solvents-organism and a suitable aerobe, a brisk fermentation is initiated in a minimum incubation period. The ideal aerobe should grow easily in corn mash and yet be susceptible to the acids produced after the carbon dioxide of the fermentation has driven all oxygen from the substrate. It should not be a spore-former, for obvious reasons.

Many types of organisms were tested to determine which are best fitted to serve this function. The aerobes which best withstand the acids and other products formed by the solvents-organism apparently bring the anaerobe out of its dormant state earliest and, in general, start the fermentations most vigorously. *Staphylococcus aureus* was chosen, as it is viable during at least the first ten hours of the fermentation. However, in all cases it is exterminated before the fifteenth hour, at which time butyl organism transfers are best made. Micro-

scopic examinations made at the twentieth hour usually fail to show the presence of any staining staphylococci whatever.

Tests have proved that the staphylococcus has no harmful effects upon the fermentation, and that it does not alter the final yield of solvents. By the use of this convenient method, much smaller amounts of spore inoculum are required. One loopful of spore suspension will give rise to fermentation when inoculated with *Staphylococcus aureus* into corn mash, while 0.5 cc. of the same suspension frequently fails to give results with the vacuum pump procedure.

Evidence to be advanced further along in this paper would indicate that the most resistant spores give rise to the most competent acetone- and butyl-producing cells. The most resistant spores of all organisms are generally supposed to have a longer lag period before germination. Therefore, it is highly important that the optimum conditions for germination should be supplied, so that *all* spores will develop as nearly simultaneously as possible. Otherwise, the non-resistant spores—the inefficient solvent-producers, will have an excellent opportunity for gaining headway and producing products inhibitory to the development of the still dormant resistant spores.

THE EFFECT OF PERIODIC "PASTEURIZATION" UPON THE YIELD OF ACETONE AND BUTYL ALCOHOL

At first, estimations of acetone yield were made by means of an adaptation of Goodwin's (1920) modification of the Messinger method. Flasks containing 150 cc. of the fermented mash, representing a definite weight of corn meal, were utilized. The flask of fermented liquor was connected to a small double-jacketed condenser and from 50 to 60 cc. of the distillate were collected in a 100 cc. volumetric flask submerged in cold water. The volumetric flask was then filled to the mark with distilled water. A 2 cc. sample of this was removed and run into a 250 cc. glass stoppered bottle containing 25 cc. of N/1 NaOH. After allowing the bottle to stand a few minutes, N/5 iodine solution was introduced in excess from a burette, with constant agitation. After standing another ten minutes the sample was rendered

acid by the addition of 25.2 cc. of N/1 H_2SO_4 and the excess iodine titrated back with N/5 thiosulphate solution, using starch paste as an indicator.

Calculations:

$$\frac{(cc.\ N/5\ I - cc.\ N/5\ Thio.) \times 0.19334}{cc.\ of\ sample} = grams\ of\ acetone$$

$$Per\ cent\ of\ acetone = \frac{grams\ of\ acetone\ (from\ above)}{weight\ of\ corn\ in\ the\ mash} \times 100$$

There is apparently no simple method for the estimation of n-butyl alcohol, but numerous investigators have observed that the ratio between acetone and butyl alcohol is always approximately one to two. This quantitative relationship was also confirmed by the writers.

A simpler method was later devised and used with success. The distillate from the 150 cc. fermentation was collected in a cooled Babcock butter-fat determination flask (for cream analysis) which had been filled to the shoulder with granular potassium carbonate. The distillation was continued until the liquid extended half way up the neck of the flask. After stoppering, the flask was placed in the incubator for a period of one hour, during which time it was agitated several times. The oily layer of mixed solvents rises to the surface and may be read in cubic centimeters from the calibration on the neck of the flask. Using the data of Reilly and Ralph (1919) on the system Water-Acetone-Butyl Alcohol, the specific gravity of a 1:2 mixture of acetone and butyl alcohol was computed. From this was derived a formula for the conversion of cubic centimeters of mixed solvents into per cent yield.

$$\frac{cc.\ mixed\ solvents}{weight\ corn} \times 80.64 = per\ cent\ yield\ of\ mixed\ solvents$$

As 7.5 gram samples of corn meal were used throughout, this becomes:

$$cc.\ mixed\ solvents \times 10.76 = per\ cent\ yield\ mixed\ solvents$$

From the fact that a negligibly small contraction occurs when

acetone and butyl alcohol are mixed, these components may be quite accurately estimated by dividing by 3.0 and 1.5 respectively.

When a series of fermentations is run, the *relative* content of solvents of each may be quite accurately gauged by the color of the fermented liquid.   In the last stage of the fermentation the butyl alcohol concentration becomes great enough to dissolve some of the otherwise insoluble zein from the corn.   This produces a golden yellow color in good fermentations.   Given a set of ten flasks containing fermented mash, it is quite simple to arrange them in order of increasing solvent content by judging the relative pigmentation in each.

The practice of heating sporulated butyl cultures was originally intended to kill any non-sporulating contaminations which might be present in the material.   It was later noted by the early workers in this field that when "non-pasteurized" starter material was used continuously, the fermentations became sluggish and the yields of solvents fell.   This was attributed to the fact that fermentations of such a type were largely the results of activities of culture developing from cells which had not passed through the rejuvenating spore stage; that is, cells which had already passed through their useful cycle.

It may be demonstrated readily that continued daily transferring of culture leads eventually to weaker and weaker fermentative action until such action practically ceases.   In such a case only a small percentage of the cells assume the spore form. Apparently the most vigorously fermenting strains are likewise the most prodigious spore-formers.   Also, a strain which is underdeveloped with respect to sporulation may be strengthened in this property by a periodic elimination of the individuals which persist in the vegetative form.   This is fortunately readily accomplished by the heat treatment.   Carrying the theory further, may not the most resistant spores give rise to the most active culture?

"Pasteurization," as practised in the following experiments, has been conducted with a view to something more than a simple destruction of remaining vegetative cells.   The heating has been

continued to a point at which some of the weaker spores are also eliminated.

A study of the effect of heat upon the viability of spores was made to determine periods of heat treatment necessary for spore destruction at different temperatures. Capillary tubes 5 cm. in length were prepared and filled with a saline suspension of *Cl. acetobutylicum* spores. The tubes were sealed and suspended in water baths of different temperatures for different periods of time. After removal, the tubes were placed for twenty-four hours in antiseptic to destroy any viable organisms adhering to the surface. Each tube was then washed and placed in a test

TABLE 7

Showing influence of heat on spores

TEMPER-ATURE	0 SEC-ONDS	10 SEC-ONDS	20 SEC-ONDS	30 SEC-ONDS	40 SEC-ONDS	50 SEC-ONDS	60 SEC-ONDS	70 SEC-ONDS	80 SEC-ONDS	90 SEC-ONDS	100 SEC-ONDS
degrees											
100	+	+	+	+	—	*	—	—	—	—	—
98	+	+	+	+	+	*	—	—	—	—	—
96	+	+	+	+	+	—	*	—	—	—	—
93	+	+	+	+	+	+	+	*	*	*	—
90	+	+	+	+	+	+	+	+	—	—	—

+ = growth within twenty-four hours; * = growth after twenty-four hours; — = no growth.

tube containing 10 cc. of *Staphylococcus aureus*-infected mash and broken by means of a sterile glass rod. (See table 7.)

Table 7 indicates that spore suspensions of this organism contain a small minority of slowly germinating individuals with a heat resistance appreciably above that of the majority. In some cases growth was not evident during the first twenty-four hours of incubation. When dealing with spore material in test tubes, forty-five seconds was found to be the limit of tolerance at 100°. Occasionally, however, if the tubes are not cooled immediately after this period, no growth will occur.

During this study over a period of two and a half years, the stock cultures were "pasteurized" on an average of once in three weeks. In each instance the tubes from which spore inoculations

were made were saved for reserve in case of contamination and also for comparison with generations past and future. Directly after "pasteurization," the spores were allowed to germinate under suitable conditions in 5 per cent mash and transplanted as an actively fermenting culture for three or four generations. The last generation was then set aside to sporulate, or else was used as a starter for a larger fermentation upon which determination of solvent content was made.

The procedure is as follows: The tube of dormant spore material from which the active culture is to be prepared is placed in a bath of boiling water for forty-five seconds. After cooling, approximately 1 cc. of the liquid suspension is removed by sterile pipette (tipless—constructed from 3 mm. tubing) and discharged, without mixing, into a test tube containing 10 cc. of *Staphylococcus aureus*-infected 5 per cent corn mash. (Best results were obtained when the mash had been inoculated with the aerobe on the preceding day.) The tube is incubated at 37° until the fermentation has proceeded to such a point that the insoluble portions of the mash begin to rise to the surface of the liquefied starch. One cubic centimeter of this is then used to inoculate a fresh tube of sterile mash. Within twenty-four hours, this tube will have reached conditions favorable for transplanting. This time, two or three drops only are used to seed a test tube of 10 cc. of mash prepared from an accurately weighed amount of oven-dried corn meal (0.5 gram). After the proper incubation period, the contents of this tube are emptied aseptically into a 300 cc. flask containing 140 cc. of sterile mash with a dry corn content of exactly 7 grams. After flaming the neck of the flask and replacing the cotton plug, the top is securely tied over with heavy paper, or better, covered with tin foil. After twenty-four hours of incubation, the insoluble portions of the mash have gathered in a slimy mat and have been buoyed up to the surface by the gas produced. The end of the fermentation is evidenced by the falling of this head to the bottom of the flask. All analyses excepting that of March 1, 1926, were made by the titration method. The acetone figure for March 1 was

TABLE 8

Showing acetone production by strain St, in series

DATE OF ANALYSIS	PER CENT ACETONE
April 15, 1924	5.60
May 1, 1924	7.90
October 1, 1924	8.15
February 1, 1925	8.60
June 1, 1925	8.90
December 1, 1925	9.30
March 1, 1926	9.30

TABLE 9

Showing acetone production by strain M

DATE OF ANALYSIS	PER CENT ACETONE
May 1, 1924	3.70
February 1, 1925	6.20
December 1, 1925	8.90
March 1, 1926	9.00

TABLE 10

Showing acetone production by strain K

DATE OF ANALYSIS	PER CENT ACETONE
May 1, 1924	3.80
February 1, 1925	5.40
December 1, 1925	8.65
March 1, 1926	9.10

TABLE 11

Showing acetone production by strain So

DATE OF ANALYSIS	PER CENT ACETONE
Ocotober 3, 1924	3.85
June 1, 1925	8.10
December 1, 1925	8.60
March 1, 1926	8.80
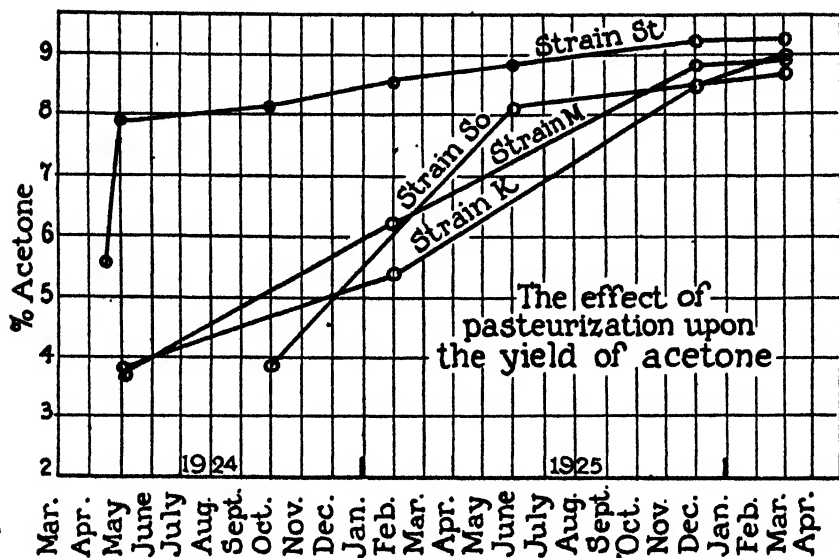
FIG. 1
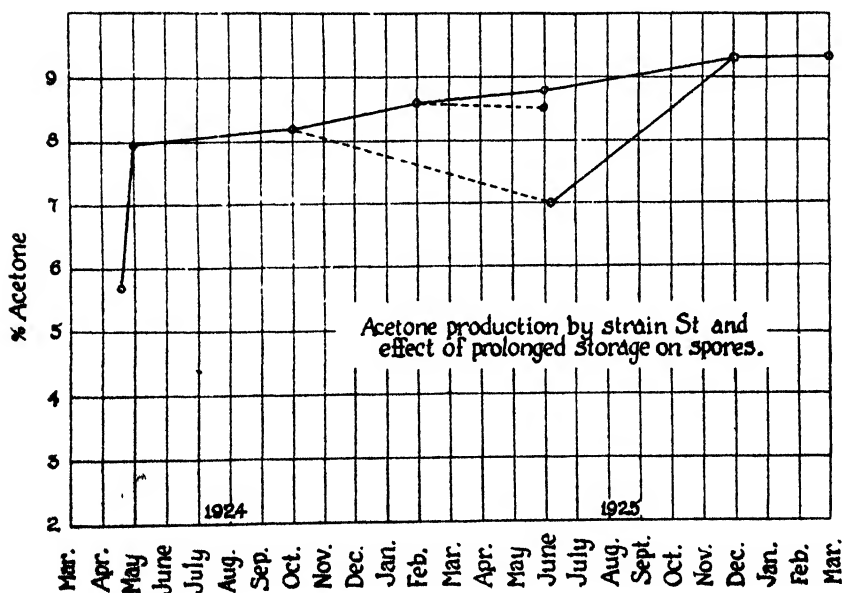
FIG. 2

computed by the salting out procedure. The increase in yield is indicated graphically in figure 1. (See tables 8, 9, 10 and 11.)

The increase in acetone yield of strains St. M, K and So is also graphically shown in figure 1. From the fact that the yields of the several strains apparently approach one another, and since the slopes of the lines have decreased to an almost dead level, we may assume that acetone and butyl alcohol production has been brought within the vicinity of the maximum.

In the commercial use of the solvents-organism, it has been customary to allow the spores to lie dormant over a period of six months before use. The effect of longer storage is shown in figure 2. A low figure is shown for mash fermented with a strain which has lain dormant for a period of eight months. The yield of acetone dropped 1.2 per cent below the former yield from the same material, and 1.8 per cent below the yield furnished by the same strain which had been subjected to periodic "pasteurizations" and frequent transfers. Spore material which was preserved over a period of four months lost little or none of its potency. In the event that prolonged storage of the sporulated material results in poor fermentations, activity may be restored to normal very rapidly by frequent "pasteurization" and transfer.

The rapid increase of acetone production by strain St. between the first and second analyses is probable evidence of rapid rejuvenation after an extended period of dormancy.

ATTEMPTS TO PREVENT CONTAMINATION AND INTERRUPTION OF THE FERMENTATION PROCESS, BY THE USE OF GERMICIDAL OR BACTERIOSTATIC AGENTS

The primary object of these experiments was to study the relative effect of various chemical inhibitory substances upon the solvents-organism and upon a common contaminating form, in an effort to discover any relationships which might be helpful in the formulation of a medium intended to exert a selective action favorable to the growth of the butyl organism. Throughout these experiments cultures contaminated with *B. mesentericus* were used. The spores of this aerobic organism are highly resistant to chemical agents and heat and are frequently en-

TABLE 12

Showing the influence of sodium chloride

	PER CENT NaCl					CONTROL
	3.0	2.0	1.0	0.5	0.3	
Solvents organism.........	−	−	−	+	+	++++
B. mesentericus.............	+	++	+++	+++	++++	++++

TABLE 13

Acriflavine

	PER CENT ACRIFLAVINE					CONTROL
	1:30T	1:60T	1:120T	1:240T	1:1,200T	
Solvents organism.........	−	−	+	+	++	++++
B. mesentericus.............	−	−	−	+	++	++++

TABLE 14

Acetone

	PER CENT ACETONE					CONTROL
	10	5	3	1.0	0.5	
Solvents organism.........	−	−	+	+	+	++++
B. mesentericus.............	−	+	+	+	+	++++

TABLE 15

Mixed oils (3:1 butyl alcohol + acetone)

	PER CENT MIXED OILS					CONTROL
	3	1	0.5	0.2	0.1	
Butyl....................	−	−	+	+	+	++++
B. mesentericus............	−	−	−	+	+	++++

TABLE 16

Showing influence of butyl resorcinol

	PER CENT BUTYL RESORCINOL					CONTROL
	1:10T	1:20T	1:50T	1:80T	1:100T	
Solvents organism.........	−	+	+++	+++	++++	++++
B. mesentericus.............	−	−	−	−	++	++++

countered in supposedly sterile mash. A medium which would aid in the purification of contaminated solvents material would be of much practical importance.

A separation of these two organisms cannot be effected by heat. Prolonged heating of mixed cultures results in the extermination of the spores of *Cl. acetobutylicum* before a material decrease in the number of viable mesentericus spores is noticed.

The result of a number of experiments in which a variety of chemical inhibitory substances were used are briefly summarized in tables 12 to 16. In general, the mixed culture used contained only spores of the two organisms. An effort was made to have the two forms represented in approximately equal numbers.

The malt extract agar gelatin medium described on page 400 was tubed in 30-cc. portions in large test tubes and sterilized. The inhibitory substances were added in concentrated form to the melted medium and thoroughly mixed. From these tubes were poured two sets of plates, one aerobic and one anaerobic. One half of the contents of each tube was poured into a Petri dish and allowed to cool; the remainder was inoculated with mixed spore suspension in each case, poured into the inverted covers of other Petri dishes, and the lower parts of the dishes floated upon the surface of the solidifying medium, as described heretofore. After streaking the aerobic plates, both sets were incubated for a period of from forty-eight to sixty hours and the colonies developing from both types of spores counted and tabulated.

The tables show representative data obtained when these and other inhibitory substances were incorporated in the malt extract agar gelatin medium. The maximum concentration of the antiseptics which allowed growth of the solvents organism generally allowed a good growth of the contaminant also.

Butyl resorcinol, however, gave more encouraging results.

Repeated experimentation with the butyl resorcinol medium showed that between the concentrations of 1:30,000 and 1:60,000 *B. mesentericus* spores practically always fail to germinate. The solvents organism, on the other hand, exhibits marked indifference to the antiseptic within this range. In the higher limits of this zone, colony formation by the anaerobe is as extensive and

the colonies are practically as large as in control plates containing no butyl resorcinol.   Pure *Cl. acetobutylicum* was recovered from

TABLE 17

0.6 per cent butyl resorcinol

	TIME					
	0	10 minutes	1 hour	4 hours	24 hours	50 hours
Solvents organism.........	+	+	+	+	+	+
B. mesentericus.............	+	+	+	+	+	−

TABLE 18

1.2 per cent butyl resorcinol

	TIME					
	0	2 minutes	10 minutes	1 hour	10 hours	24 hours
Solvents organism.........	+	+	+	+	+	+
B. mesentericus.............	+	+	+	+	+	−

TABLE 19

2.5 per cent butyl resorcinol

	TIME				
	0	2 minutes	10 minutes	24 hours	48 hours
Solvents organism................	+	+	+	+	−
B. mesentericus....................	+	+	−	−	−

TABLE 20

5.0 per cent butyl resorcinol

	TIME			
	0	2 minutes	10 minutes	24 hours
Solvents organism..................	+	+	+	−
B. mesentericus....................	+	+	−	−

contaminated spore material by picking surface colonies from a 1:50,000 butyl-resorcinol plate which had been incubated in an anaerobic jar.

The relative germicidal action of butyl resorcinol on the spores of the solvents organism and *B. mesentericus* was also determined.   Mixed spore suspensions in butyl resorcinol solutions of varying concentrations[2] were incubated for different periods of time and loopfuls of the suspension used to inoculate *Staphylococcus aureus*-infected mash.   After a twenty-four-hour incubation period, the presence of characteristic butyl fermentation indicated the survival of the butyl spores in the germicidal solution.   Streaking agar plates from the incubated mash revealed the presence of *B. mesentericus* when it survived the butyl resorcinol solution.   (See tables 17 to 20).

The experiments dealing with butyl resorcinol as a germicide (for spores) as indicated in the tables, give further proof of the selective action of this substance.   Spores of *Cl. acetobutylicum* exposed to a 2.5 per cent butyl resorcinol solution for twenty-four hours retain their viability, but those of *B. mesentericus* are killed in the same solution, in the same test tube, in ten minutes.   This may furnish another method for the separation of solvents-bacillus spore material from this contaminant.

The selective action of butyl resorcinol can doubtless be attributed to the natural resistance which the butyl organism must presumably possess against the butyl radical.   The value of butyl resorcinol as a general germicide is due in large part to the presence of the butyl radical, as comparative tests of resorcinol and the butyl substitution product indicate.

SUMMARY AND CONCLUSIONS

Six strains of butyl alcohol and acetone-producing organisms were studied.   Four of these were newly isolated, and two were obtained from other laboratories.   No qualitative differences in physiological properties were noted.   These similarities and the ready agglutinability of all strains with antisera prepared by the immunization of rabbits with killed cultures point definitely to the conclusion that the organisms studied here, which are

[2] Butyl resorcinol is not very soluble in water.   In the stronger concentrations, a few drops of alcohol were added to render the germicide miscible with water.

responsible for large scale production of butyl alcohol and acetone, constitute a definite species with clear-cut characteristics.

The organism is characterized by powerful diastatic, saccharolytic and proteolytic activities. All of the common and most of the rarer sugars are sources of available carbon. Glycerol, regarding which there have been conflicting reports, was not attacked in these experiments.

Experiments on nitrogen requirements indicate that the organism must have a complex nitrogen supply which is furnished by proteins and commercial peptones. Ammonium salts, the availability of which has been disputed, were found not to induce growth. The negative results obtained with the amino acids offer some evidence that simple amino acids, at least of the types used here, fail to support growth.

By the addition of an aerobe, as for example, *Staphylococcus aureus*, to sterile mash prior to, or simultaneously with, the introduction of "pasteurized" spores of the solvents organism, suitable conditions of anaerobiosis are produced for the germination of the spores. This method has proved more convenient, more economical and more efficient than the customary vacuum method.

Estimation of mixed solvent content by the salting-out procedure as described in this paper has proved to be more convenient and rapid than the Messinger titration method.

The almost continued culturing of *Cl. acetobutylicum* in 5 per cent maize mash, with periodic "pasteurization" after complete sporulation, has during a period of two years resulted in an average increase in solvent production of over 100 per cent for the four strains so treated. The storage of spores for periods of over six months apparently decreases the solvents producing power. Such stock strain may be rejuvenated, however, by alternate "pasteurization" and subculturing.

A study of the effect of various germicidal and antiseptic substances upon both the vegetative form and the spore of the solvents-producing organism shows this unique anaerobe to be comparatively susceptible to such agents. Exception is found, however, in its reaction to butyl resorcinol, which is far less toxic

to this organism than to such resistant and hardy forms as members of the subtilis-mesentericus group. This property may be attributed to the naturally high resistance of the solvents-producing species to the butyl radical which is responsible for the high toxicity of this compound toward other organisms generally.

REFERENCES

AVERY, O. T., AND MORGAN, H. T. 1923. Artificial peroxidase and growth of anaerobic bacteria. Proc. Soc. Exp. Bio. Med., 21, 59.

GOODWIN, L. F. 1920 Modification of the Messinger method for acetone determination. Jour. Amer. Chem. Soc., 42, 39.

GRUBER, M. 1887 Eine Methode der Kultur anaërobischer Bacterien. Cent. f. Bakt., 1, 367.

KRUMWIEDE, C., JR., AND PRATT, J. 1913 Fusiform bacilli. Isolation and culture. Jour. Inf. Dis., 12, 199–201.

McCOY, ELIZ., FRED, E. B., ET AL. 1926 A cultural study of the acetone-butyl alcohol organism. Jour. Inf. Dis., 39, 457–483.

REILLY, J., AND RALPH, E. W. 1919 The system Butyl Alcohol-Acetone Water. Proc. Royal Society Dublin, 15, 597.

WEIZMANN, C. 1919 Production of Acetone and Alcohol by a bacteriological process. U. S. Patent No. 1,315,585.

DIFFERENTIAL STAINING AS A CRITERION OF THE VIABILITY OF BACTERIAL SPORES[1]

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REVIEW OF LITERATURE

The differentiation of living from dead bacterial cells was studied by Metchnikoff as early as 1896, according to Seiffert (1922) who gives a brief review of what had been accomplished along this line by prior investigators. Henrici (1923) does not mention specifically the bacteria studied by him, but his observations are nevertheless worthy of note. Heat-killed cells stained while living cells did not; but with formalin as a killing agent he was unable to get any staining. Henrici also noticed that the staining was not as intense immediately after the heating as it was after several hours had elapsed, and that the cells stained more readily after heating to 60 degrees than when heated to 100 degrees. It is my opinion that the time factor is probably accountable for this difference, those spores killed by heating to 60 degrees being exposed for a longer period.

In 1923, Burke, working with the spores of *Cl. botulinum*, developed a method of differential staining for living and dead bacterial spores. Her most intensive work was with the anaerobe mentioned, but she also tested the method with *B. anthracis*, *B. subtilis*, *B. vulgatus*, and *B. cereus*, and seemed satisfied with the results. The smears were air-dried to avoid heating, and then stained by immersion in a beaker of steaming carbol-fuchsin for two minutes. The excess dye was washed off with water, then with absolute acetone; the acetone was removed with water, and

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[1] Thesis presented in partial fulfilment of the requirements for the Certificate in Public Health of Yale University.

the preparation was counterstained with Loeffler's methylene blue for two minutes. Koser and Mills (1925) found that if they followed precisely Burke's technique with the spores of *B. megatherium* more than half of the cells stained solidly, i.e., as if they were dead. Such a mortality rate in a young spore suspension was unnatural as indicated by the work of Swann (1924). Koser and Mills, therefore, proceeded to work out the time and temperature factors for the staining, and found that one or two minutes in carbol-fuchsin, at room temperature, gave satisfactory results with *B. megatherium*, *B. cereus*, *B. subtilis*, *B. mesentericus* and *B. ramosus*, but not with *B. terminalis* and *B. fusiformis*. These last two species they were unable to stain differentially.

The object of the present investigation was to test further the reliability of the Burke method, as modified by Koser and Mills and, in particular, to see whether this staining procedure will distinguish between living and dead spores when death has resulted from some cause other than high heat.

MATERIAL USED IN THE PRESENT STUDY

Although the heat resistance of the spore of *Cl. botulinum* has probably been studied more exhaustively than that of any other spore, it was not thought wise, in the short time available for the study here reported, to make use of a spore which may germinate after a year's incubation. The organism used throughout was therefore a stock laboratory strain of *B. cereus*. The study by Winslow and Brooke (1927) gives evidence that the vegetative cells of this organism die out quite rapidly in water unless in the presence of protective colloids. A day was therefore allowed for the dissolution of any vegetative cells in the suspension, which was adequate time as shown by the fact that plate counts on successive days for over a week revealed no further decrease in the number of viable cells present. A suspension was prepared by washing off the surfaces of three agar slant cultures with sterilized tap water into a 75 cc. test tube. Absorbent cotton filters, made by plugging tubes of slightly smaller bore with cotton rolled so that there were no creases through which streams of the suspension could leak, had been previously sterilized. One of these balls was pushed

into the tube containing the wash from the agar slants so that it fitted tightly; the liquid was then forced up through it by pushing the cotton down, and thus the suspension was strained of its clumps. The presence of clumps would not only make the stained preparations harder to count, but also as has been shown by Harrison and Hood (1923), clumps of spores cause inconsistencies in the results of heating experiments. The strained suspension was then poured off into a sterile container and adjusted to a turbidity between nephelometer values of 1.5 and 2.

STAINING PROCEDURE

The same staining solutions were used throughout. The carbol-fuchsin was made up as follows: 1.5 grams of basic fuchsin dissolved in 15 cc. of 95 per cent alcohol was added to 5 cc. of melted phenol crystals in 85 cc. of water. The Loeffler's methylene blue was the usual laboratory solution: 30 cc. of saturated alcoholic solution of methylene blue in 100 cc. of 1:100,000 aqueous sodium hydroxide. The suspension placed on the slide was allowed to air-dry thoroughly before staining. The procedure for staining was as follows: after immersion in the carbolfuchsin at room temperature for two minutes, the surplus dye was washed off with water, then followed a moment's decoloration with acetone, which was washed off with water before immersion in the methylene blue for two minutes. The slides were drained dry in preference to blotting. The examination of the slides was always made by the same microscope set up, and by artificial light. Slides prepared from a suspension heated to sterility showed only spores stained solidly red, the vegetative rods staining blue. In an unheated suspension, the majority of spores showed up as red ring forms, with only a few spores staining solidly. Believing that in the practical application of this method a doubtfully viable organism was as good as a definitely viable organism, a ring stained form was counted as a ring form, regardless of the shade of pink in the center. This is a different line of division than that chosen by Burke, but would not make any perceptible difference in the counts, except in the moderate heating experiment.

In order to determine the reliability of the counting procedure employed and the number of cells which must be counted to ensure comparability, a series of check determinations was made on a single mixture containing approximately equal proportions of a suspension of heat-killed spores and a suspension of untreated spores. When 250 cells were counted the percentage of ring-forms (living spores) recorded was 44; when 500 cells were counted it varied from 44 to 48 in four trials; when 1000 cells were counted it was 47. It would seem then that the counting of 250 cells will yield results within the 10 per cent range of variability attained by ordinary plating methods. In all my work, I counted 500 cells.

It is important, however, to make the counts in the central part of the preparation, since the debris at the edges causes solidly staining spores to be missed. Thus in a special study of this point it was found that counts at the edge gave 63 to 69 per cent of ring forms as against 52 per cent recorded for the central fields.

PLATE COUNTS

Considerable difficulty was experienced in determining the number of viable organisms in a suspension by plate counts. The ordinary Petri dish is too small. The Kolle flask is better, but even here spreaders are hard to avoid. The 10 cm. Petri dish was found to be the best form of plating apparatus, but the colonies of B. cereus are so large that only 75 to 100 can be obtained as separate colonies on one plate. The results obtained, however, were concordant within the normal limits of error and plate counts and staining results check rather closely. Thus, in an untreated suspension held for eight days and observed daily, the count of viable spores by the plate method varied from 6,250,000 to 10,000,000 per cubic centimeter, while the proportion of ring forms under the microscope varied from 97.8 to 99.5 per cent.

STUDIES ON CELLS KILLED AT 122°C.

My first studies were made by the method employed by Koser and Mills. A standard suspension of viable cells was prepared

and divided into portions.   One portion was untreated, while the other was treated in the autoclave for twenty minutes under 15 pounds pressure.   (The total period in the autoclave was about fifty minutes).   Observations were then made on (a) the unheated suspension, (b) the heated suspension, and (c) a 1.1 mixture of

TABLE 1

Spores exposed to steam at 15 pounds pressure for twenty minutes

	VIABLE CELLS PER CUBIC CENTIMETER BY PLATE COUNT		PER CENT VIABLE CELLS (RING FORMS) BY MICROSCOPIC COUNT
	Number	Per cent	
Unheated suspension..	28,000,000	100	99
Heated suspension......	8,400	0 03	0
1.1 mixture of heated and unheated suspension...........	15,650,000	56	49

TABLE 2

Spores exposed to steam at 11 pounds pressure for a moment

	VIABLE CELLS PER CUBIC CENTIMETER BY PLATE COUNT		PER CENT VIABLE CELLS (RING FORMS) BY MICROSCOPIC COUNT
	Number	Per cent	
Unheated suspension...........	8,000,000	100	99
Heated suspension.........	43,750	0.5	1-2
1.1 mixture of heated and unheated suspension	3,125,000	39	59-61

TABLE 3

Spores exposed to direct sunlight for varying periods

	PERIOD				
	0 hours	14 hours	20 hours	27 hours	35 hours
Per cent ring forms	99	94	89	83	79

the two suspensions.   Results of a single typical experiment are presented in table 1.

STUDIES ON CELLS KILLED AT 117°C.

My next studies were made to determine the effects of more moderate heating.   In these tests the spores were exposed to

steam at 11 pounds pressure for as brief a period as possible, the total time in the autoclave being twenty minutes, ten minutes for increasing the pressure and an equal period for decreasing it.

The general results of one such experiment are presented in table 2.

Here, it will be noted that the percentage of ring forms is substantially larger than the number of viable cells as indicated by the plate count. Furthermore, it was obvious on inspection that the microscopic slides showed a great many cells exhibiting an intermediate degree of staining, varying from clearly solid forms through various stages of dark and light centers to definite rings with transparent interiors. These slides were exceedingly difficult to count with accuracy.

STUDIES ON SPORES EXPOSED TO SUNLIGHT

The next observations were made on spores dried on slides and then exposed to the direct rays of the sun for seven hours a day from 8:30 a.m. to 1 p.m. and again from 2 p.m. to 4:30 p.m. This experiment was performed in April when the ultra-violet rays of sunlight are of about an average intensity according to Hess.

The results of the microscopic observation of slides thus exposed for two, three, four and five days are presented in table 3. Check slides held dry in the dark for corresponding periods showed no decrease in ring forms and the heating effect of the sun while these experiments were conducted was practically negligible.

In this experiment no intermediate forms were noted, the cells appearing either as clear rings or as fully stained circles. It will be noted that the percentage of ring forms steadily decreases; but it does not decrease nearly as fast as one might expect. These tests were not controlled by plate counts but somewhat similar tests by Weinzirl (1914) showed that five different types of spore-forming organisms were completely destroyed by exposure to sunlight for two to eight hours.

STUDIES ON SPORES EXPOSED TO CHEMICAL DISINFECTANTS

Finally, the same technique was applied to spores exposed in suspension for varying periods to acid, alkali, ethyl alcohol and

mercuric chloride. Gentian violet was also studied but left the spores all viable in the concentrations tested.

The plate count results obtained by these tests showed in general a decrease of 55 to 65 per cent in viable cells and in one instance (after twenty-four hours' exposure to 1:4000 HgCl₂) a decrease of 96 per cent. Yet in no case was the proportion of solidly staining cells in the microscopic preparation substantially increased. This observation is in accord with the finding of Koser and Mills (1925) for mercuric chloride.

SUMMARY AND CONCLUSIONS

1. Burke's differential staining method for living and dead bacterial spores in general stains solidly spores killed by heat. Koser and Mills' modification of Burke's technique gives an even closer correlation of the differential count with the probable death rate in aerobic spore suspensions than does the original Burke method; but even with this procedure it appears that, when spores are exposed to moderate heat, loss of viability precedes the changes which cause solid staining.

2. Exposure to the direct rays of the sun causes a definite increase in the proportion of spores which stain solidly, but the proportion of ring forms still remains much higher than would be expected according to observations of Weinzirl in regard to viability under similar conditions.

3. Although alcohol, sodium chloride, hydrochloric acid, sodium hydroxide and mercuric chloride cause a marked loss of viability, there seems to be no corresponding decrease in the per cent of ring staining forms.

4. Spores which stain solidly by the method here described may safely be assumed to be dead; but among spores which still show the ring staining, there may be many which will no longer grow on solid culture media.

5. The most probable explanation of these phenomena would seem to lie in the assumption that the death of the cell precedes those chemical or physical changes which affect its stainability; while in the case of certain chemical disinfectants, the lethal process may be such as to leave the staining reactions substantially unaffected.

REFERENCES

BAYNE-JONES, S., AND VAN DER LINGEN, J. S. 1923 Bactericidal action of ultra-violet light. Johns Hopkins Bull., **34**, 11–15.

BAZZONI, C. B. 1914. Destruction of bacteria through the action of light. Amer. Jour. Public Health, **4**, 975–992.

BIGELOW, W. D., AND ESTY, J. R. 1920 Thermal death point in relation to time of typical thermophylic organisms. Jour. Inf. Diseases, **27**, 602–617.

BREED, R. S., AND BREW, J. D. 1916 Counting bacteria by means of the microscope. N. Y. Agric. Exper. Station, Technical Bull. No. 49.

BURKE, G. S. 1923 Studies on the thermal death time of spores of *Clostridium botulinum*. 2. Differential staining of living and dead spores. Jour. Inf. Diseases, **32**, 433–438.

BURKE, V., AND SKINNER, H. 1925 Resistance of bacterial spores to triphenylmethane dyes. Jour. Exper. Med., **41**, 471–477.

CHICK, H. 1908 An investigation of the laws of disinfection. Jour. Hyg., **8**, 92–158.

CHICK, H. 1910 Process of disinfection by chemical agencies and hot water. Jour. Hyg., **10**, 237–286.

CHICK, H. 1912 Factors conditioning the velocity of disinfection. Eighth Inter. Cong. Appl. Chem., **26**, 167–197.

CHURCHMAN, J. A. 1923 Mechanism of bacteriostasis. Jour. Exper. Med., **37**, 543–551.

DICKSON, BURKE, BECK, JOHNSTON AND KING 1922 Studies on the thermal death time of spores of *Clostridium botulinum*. Jour. Amer. Med. Assoc., **79**, 1239–1240.

DOWNES, A., AND BLUNT, T. P. 1877 Researches on the effect of light upon bacteria and other organisms. Proc. Roy. Soc. of London, **26**, 488–500.

ESTY, J. R., AND WILLIAMS, C. C. 1924 Heat resistance studies. 1. A new method for the determination of resistance for bacterial spores. Jour. Inf. Diseases, **34**, 516–528.

GRAHAM-SMITH, G. S. 1910 Division and post-fission movement of bacilli when grown on solid media. Parasitology, **3**, 17–53.

HALL, I. C. 1919 Selective elimination of hay bacillus from cultures of obligative anaerobes. Jour. Amer. Med. Assoc., **72**, 274.

HARRISON AND HOOD 1923 Trans. Roy. Soc. of Canada, **17**.

HENRICI, A. T. 1923 Differential counting of living and dead cells of bacteria. Proc. Soc. Exper. Biol. and Med., **20**, 293–295.

HERTEL, E. 1904 Ueber Beeinflussung der Organismus durch Licht, speziel durch die chemisch wirksamen Strahlen. Zeit. f. allgem. Physiol., **4**, 1–43.

HESS, A. F. 1925 The ultra-violet rays of the sun. Jour. Amer. Med. Assoc., **84**, 1033–1037.

KOSER, S. A., AND MILLS, J. H. 1925 Differential staining of living and dead bacterial spores. Jour. Bact., **10**, 25–36.

LAWRENCE, J. S., AND FORD, W. W. 1916 Studies on aerobic spore-bearing nonpathogenic bacteria. Jour. Bact., **1**, 273–319.

MAGOON, C. A. 1926 Studies upon bacterial spores. 1. Thermal resistance as affected by age and environment. Jour. Bact., **11**, 253–283.

ROBERTSON, A. H. 1921 Relation between bacterial counts from milk as obtained by microscopic and plate methods. N. Y. Agric. Exper. Station Tech. Bull. No. 86.

SEIFFERT, W. 1922 Vergleichende Farbersuche an lebende und toten Bakterien. Centralbl. f. Bakt., Abt. I, Orig., **88**, 151–158.

SWANN, M. B. R. 1924 On the germination period and mortality of the spores of *Bacillus anthracis*. Jour. Path. and Bact., **27**, 130–134.

TILLEY, F. W., AND SHAFFER, J. M. 1926 Relation between chemical constitution and germicidal activity of monohydric alcohols and phenols. Jour. Bact., **12**, 303–309.

WEINZIRL, J. 1914 The germicidal action of sunlight upon bacterial spores. Amer. Jour. Pub. Health, **4**, 969–974.

WEISS, H. 1921 The heat resistance of spores with special reference to the spores of *B. botulinus*. Jour. Inf. Diseases, **28**, 70–92.

WILSON, G. S. 1922 The proportion of viable bacteria in young cultures with special reference to the technic employed in counting. Jour. Bact., **7**, 405–446.

WINSLOW, C.-E. A., AND BROOKE, O. R. 1926 Viability of various species of bacteria in aqueous suspensions. Jour. Bact., **13**, 235.

EMPLOYMENT OF A DOUBLE SUGAR MEDIUM FOR ROUTINE DIAGNOSIS OF BACILLARY WHITE DIARRHEA, FOWL TYPHOID, AND FOWL CHOLERA[1]

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The ordinary procedure in diagnosing the common poultry diseases consists in making cultures on agar slants and, after growth has appeared, transferring the culture to four fermentation tubes containing respectively lactose, glucose, sucrose, and either maltose, dextrin, or dulcitol. The fermentation of glucose alone, either with or without the production of gas, indicates the presence of *Salmonella pullorum*, the cause of bacillary white diarrhea. If both glucose and maltose (dextrin or dulcitol may be used in place of maltose) are fermented, but neither lactose nor sucrose, the organism is *Salmonella gallinarum*, the cause of fowl typhoid. Acid in glucose and sucrose, but not in lactose or in maltose, shows the organism to be that of fowl cholera (*Pasteurella avicida*).

Hadley and his co-workers (1918) showed that *Salmonella pullorum* and *Salmonella gallinarum* produce approximately the same amount of acid in glucose. Most cultures of *S. pullorum* produce gas in glucose. *Pasteurella avicida* ferments glucose, but not as strongly as *S. pullorum* and *S. gallinarum*. These workers also demonstrated that maltose is fermented more rapidly and with greater acid production by *S. gallinarum* than are either dextrin or dulcitol. *S. pullorum* produces at once an alkaline reaction in maltose, whereas *Pasteurella avicida* may or may not produce a very slight acid fermentation of this sugar. In any case the reaction is never alkaline. The same applies to dextrin and dulcitol.

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[1] Contribution No. 351 of the Rhode Island Agricultural Experiment Station.

The above information led the author to prepare the following medium, having in mind the saving of both time and expense. Two formulas are given which show equally good reactions.   The idea of a double sugar medium is not at all new.   Russell (1911) and others have applied such a method to the differentiation of fecal organisms.   In the preparation of such a medium it is desirable to have present, growth-producing materials, in proportions which will be conducive to the production of gas by *Salmonella pullorum* .Goodner and May (1927) suggest the following foundation which proved to be entirely satisfactory and is used as the basis of one of the formulas:

Peptone	10 parts
Meat extract	5 parts
Sodium chloride	10 parts
Water	1000 parts

To the above is added 15 parts of agar.   The materials are heated to dissolve them, and the medium is autoclaved and filtered through cotton to clarify it.   While the medium is still hot, one part glucose, five parts maltose, and ten parts Andrade's indicator are added.   The reaction is adjusted so that the medium is a bright red when hot and practically colorless when cold.   In other words the reaction should be as nearly acid as the use of this indicator will permit.   The complete medium is now tubed to a depth of about two inches and sterilized in the autoclave at 10 pounds pressure for fifteen minutes.   When sterile, the tubes are slanted so as to get a deep butt.   Inoculations are made by surface streak and stab.

The second formula to be successfully used has for a basis the following constituents:

Proteose peptone (Difco)	10 parts
Sodium chloride	10 parts
Agar	15 parts
Water	1000 parts

The medium is clarified by autoclaving and filtering, the sugars and indicator are added as above, the reaction is adjusted, and the medium is tubed, sterilized, and slanted as in the case of the

first formula. It will be noted that proteose peptone is used in place of the peptone ordinarily employed. and that the meat extract is omitted from the second formula. Comparative tests showed that the presence of meat extract did not materially aid gas production in this medium, while without it there was a more marked color reaction.

The method used for diagnosis of the three poultry diseases under consideration is as follows:

Cultures from the various organs are streaked on Lactose Andrade Agar slants consisting of infusion agar to which has been added 1 per cent lactose and 1 per cent Andrade's indicator. The reaction is adjusted as before mentioned. The slants are incubated at 37 degrees for twelve hours or more and examined. If lactose is fermented or if no growth occurs the test is negative for any of the three mentioned diseases. If growth occurs and lactose is not fermented, a Gram stain is made of the organism. Gram-negative, non-spore-forming rods are inoculated into tubes containing one of the double-sugar media described above, and sucrose respectively. The lactose serves to eliminate coli-like organisms while the sucrose detects the organism of fowl cholera. The double sugar medium differentiates between *S. pullorum* and *S. gallinarum*. The readings depend on the ability of *S. pullorum* to produce gas in glucose, and on the immediate slight reduction of acidity in maltose. *S. gallinarum* shows strong acid producing powers in both glucose and maltose. This organism produces a permanent deep red color in both the slant and butt of the double sugar medium in eighteen to twenty-four hours. *Salmonella pullorum*, on the other hand, usually produces gas in the butt with a colorless slant and a faint pink butt. Sometimes, however, the faint color appears on both slant and butt. If one is dealing with an organism of the so-called anaerogenic type this might prove confusing. In such a case the tube is incubated for twelve to twenty-four hours longer. If the organism in question is *S. pullorum* the medium will show almost complete decolorization. If incubated longer the color entirely disappears. *Salmonella gallinarum* retains its color over an observed period of two weeks. If the organism is that of fowl cholera no gas is produced in the

double-sugar medium, but the butt is colored a faint pink. The acid produced is insufficient to color the slant. The tube of sucrose turns red, whereas with *S. pullorum* and *S. gallinarum* no change occurs. Several writers have reported irregular results on the reaction of *S. pullorum* in maltose. May and Goodner (1927) have shown that these slight positive reactions in maltose are due to the fact that this sugar is easily broken down in the process of sterilization. They report that sterilization in the autoclave at 10 pounds produces less hydrolysis in maltose than sterilization in the Arnold. In the medium under discussion the power of *S. pullorum* to produce an alkaline reaction in maltose is sufficient to counteract in 48 hours any slight acid fermentation of the products of the hydrolysis of maltose. If gas is produced the organism is, in any case, *Salmonella pullorum*.

Tests conducted on nearly two hundred known and unknown cultures showed this method to be more satisfactory than the usual cultural methods. Many cultures which failed to produce gas, or did so very slowly, in the ordinary glucose fermentation tube, showed gas bubbles in the double sugar medium here described. In any case the decolorizing action of *S. pullorum* served as a check on the readings.

There was no apparent difference either in color or in gas production if the amount of maltose was increased to one per cent. Larger amounts of glucose than 0.1 per cent could not be used because of confusing color reactions. With amounts of glucose in excess of 0.3 per cent there was no difference in the amount of color produced by *S. pullorum* and *S. gallinarum*. Here, gas production was the only criterion.

SUMMARY

A method for the routine differentiation of bacillary white diarrhea, fowl typhoid, and fowl cholera is given by which the cost of material and labor is considerably reduced. The method requires about half the number of transfers usually employed, and slightly less time is consumed in making a diagnosis. In nearly two hundred controlled tests with known and unknown cultures the method gave clear cut reactions. It consists in the

inoculation of Gram-negative non-spore-forming rods which do not ferment lactose, into a medium containing 0.1 per cent glucose and 0.5 per cent maltose.   A tube of one per cent sucrose broth serves to detect the organism of fowl cholera.

REFERENCES

GOODNER, K., AND MAY, H. G.   1927   The production of gas by *Salmonella pullorum*.   R. I. Agr. Exp. Sta. Bul. 208.

HADLEY, P., ELKINS, M. W., AND CALDWELL, D. W.   1918   The colon-typhoid intermediates as causative agents of disease in birds: I. The paratyphoid bacteria.   R. I. Agr. Exp. Sta. Bul. 174, 216 p.

MAY, H. G., AND GOODNER, K.   1927   Cultural and antigenic studies on *Salmonella gallinarum* and *Salmonella pullorum*.   Jour. Bact., **13**, 129–146.

RUSSELL, F. F.   1911   The isolation of typhoid bacilli.   Jour. Med. Res., **25**, 217–229.

A PRACTICAL METHOD OF CULTURE FROM A SINGLE BACTERIAL CELL

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The importance of obtaining pure line strains of bacteria, so often essential, especially where genetical considerations or questions of physiological identity are concerned, has only recently been recognized. To obtain a single cell for the culture of a bacterium is by no means so easy a task as to obtain a single fungal spore or yeast cell.

Several methods have been described of which the "Chinese ink Method" of Burri (1909) is the most popular. The method is excellent in that one actually sees the silhouette of the single cell and has an opportunity of watching its development into a colony; it is however a very tiresome and tedious technique. The "mercury shield" method of Barnard (1921, 1925) is no doubt an excellent method in the hands of Barnard, but it requires most delicate manipulation and is hardly one which could ever come into ordinary laboratory practice. The latest method, that of Dickinson (1926), uses a piece of apparatus designed by the author and executed by Messrs. Leitz. With this instrument he claims that it is quite possible to isolate a single bacterial cell, even of the smallest dimensions; whether this can be done with sufficient ease to make the operation practicable the writer is not in a position to say, but his experience is enough to show him that, even if workable, the task for any but the largest forms of bacteria would be an extraordinarily tedious one.

One feels the need of a rough and ready method which can be put into everyday practice and which will hold out a ninety-nine per cent chance that the culture is pure. It is believed that the

method herein described fills this need. It is a modification of the old method of separation by attenuation. The attenuations are made in sterile water in a series of five sterile watchglasses. A plate of nutrient agar with the bottom of the dish marked by Chinese ink with nine rows of about 10 spots in each row, is placed near by. The suspensions are then examined by placing a loopful of each in turn under a cover-slip and viewing with the microscope to see that the organisms are not clumped and to form an opinion as to the density of the suspensions. From this inspection, with a little experience, one can judge which are the appropriate suspensions to employ. With a steel mapping pen, sterilized by alcohol and flaming, the weakest of three selected suspensions is taken and spotted on the surface of the agar to cover three of the marked rows, one drop being placed near to, but not quite exactly over, each of the ink spots. By just lightly touching the agar the droplet can be made so small that it will easily come within the field of a 2/3 objective, and usually one filling of the pen is sufficient to cover the whole of the thirty spots in the three rows.

The plates after inoculation are incubated over-night at a temperature suitable for the organism, and are examined with the low power of the microscope; the dish being placed bottom up, the position of each colony is found by searching the surface of the agar in the neighborhood of the ink spot on the bottom of the dish. If the plating has been satisfactorily carried out one finds that each spot, representing the strongest of the chosen suspensions, shows many initial minute colonies, say fifteen to twenty, which will later coalesce to form one colony. It seems quite clear under the microscope that most of these initial spots arise from a single cell. Passing down the three rows representing the second of the chosen suspensions one finds the initial spots reduced to a number of one to four with here and there a blank. Then the rows representing the greatest dilution will contain, for the most part, blanks with here and there a colony with one point of origin or possibly two points but no more. Now it is easy to see that all these colonies which one marks down as having arisen from one point, develop during the next few hours into

colonies of precisely the same shape and size. Any in which one detects two points develop into colonies of greater size. One therefore feels that by selecting colonies marked as having one point of origin and neglecting any of these which subsequently develop to more than minimal size, one can confidently rely on such colonies having developed from single cells.

REFERENCES

BARNARD. 1925. Brit. Jour. Exper. Path., **6**, 39.
BARNARD, TOPLEY AND WILSON. 1921–22. Jour. Hygiene, **20**, 221.
BURRI. 1909. Das Tuscheverfahren, Jena.
DICKINSON. 1926. Roy. Soc. Med., **19**, 1.

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